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The Journal of Biochemistry, Vol. 45, No. 8, 1958

CRYSTALLINE CYTOCHROME C

I. CRYSTALLIZATION OF MAMMALIAN HEART CYTOCHROME C

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(Received for publication, December 12, 1957)

Numerous methods have so far been published for the preparation of cytchrome c from mammalian hearts, especially from beef and horse hearts. The extraction of the cytochrome with an acid was first employed by Theorell (*1*). Keilin and Hartree (*2*) purified the protein by precipitation with TCA* in AS solution. For further purification, elctrophoresis was used by Theorell and Akesson (*3*) and the removal of impure proteins with saturated AS by Keilin and Hartree (*4*). Paléus and Neilands (*5*) introduced chromatography on the cation exchange resin, Amberlite IRC-50, and the method was further improved by Margoliash (*6*). Thanks to these excellent investigations, it is now very easy to obtain practically pure preparation of cyt. c from mammalian hearts. In spite of the fact that all these investigations were mainly concerned with mammalian cyt. c, the first crystallization of this cytochrome was accomplished from muscle of king penguin by Bodo in 1955 (*7*). Details of the purification procedure have, however, not yet been reported. There is little doubt that mammalian heart, especially beef heart, is the most convenient source of cyt. c, since the material is easily available and the yield of the pigment is considerably high.

In the previous reports, we reported on a method applicable for preparing crystalline cyt. c from beef and pig hearts (*8*, *9*) and horse heart (*9*) as well as from yeast (*9*, *10*). This method consisted in the extraction with AS solution at neutral pH, TCA-AS precipitation at pH 4.5–5, adsorption on an ion exchange resin followed by elution, removal of impurities with nearly saturated AS, chromatography on the resin, concentration by the resin column and crystallization of reduced cyt. c in AS solution. A few steps of the above method have now been improved in the following manners: (a) the pigment was extracted with AS solution after the treatment of tissue with acetic acid, (b) the washing in the adsorption process was improved, and (c) the removal of impurities with AS was omitted. Thus, the method has been fairly simplified and the yield of the crystals considerably increased. The present paper describes the details of the improved method.

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* The Following abbreviations are used in this paper: TCA for trichloroacetic acid, AS for ammonium sulfate, cyt. c for cytochrome c, and Am XE-64 for Amberlite XE-64.

EXPERIMENTAL

Assay Method

The concentration and purity of cyt. c were spectrophotometrically determined after reduction with sodium dithionite at pH 6.5 to 8.0. For the calculation of the concentration, molecular extinction coefficient of cyt. c at 550 mμ, $\varepsilon_{550}=27.7\times 10^3\,M^{-1}$ cm.$^{-1}$ (*11*), was used. The purity of cyt. c was represented by the ratio of the extinction of the reduced form at 550 mμ to that of the oxidized form at 280 mμ.

Preparation of Resin

One kg. of the resin, Am XE-64, was well suspended in 10 liters of tap water, and the supernatant suspension was removed after settling for 3 hours. The same settling process was repeated three more times in order to sufficiently remove fine particles of resin. The settled resin was suspended in 5 liters of 2 *N* sodium hydroxide and the suspension was heated at 80° to 90° for 1 hour with occasional stirring. The supernatant was decanted off after settling for 3 hours and the resin was washed twice with each 10 liters portion of water. The settled Na-form resin was further suspended in 5 liters of water and passed through a 200 mesh sieve by the aid of occasional jet of tap water on the sieve. The resin passed through the sieve was settled, about 0.1 volume of 5 per cent sodium hypochlorite was mixed with the resin, and the heavy suspension was allowed to stand overnight. The resin, which now turned to be quite colorless, was transferred into a large glass filter and washed by passing 10 liters of water. The resin was suspended in 5 volumes of 2 *N* hydrochloric acid, collected on a glass filter, and then washed with 10 liters of tap water followed by 5 liters of distilled water. After the resin was used, it was regenerated by the treatment with NaOH, NaOCl and HCl as above.

The ammonium phosphate buffer containing 0.25 g. ion of ammonium ion* per liter (hereafter referred to as 0.25 *N*) of pH 7.0±0.1 was prepared by mixing 0.125 *M* $(NH_4)_2HPO_4$ and 0.25 *M* $NH_4H_2PO_4$ solution. The resin was equilibrated with this buffer in a way similar to that reported by Hirs *et al.* (*12*): The water-washed H-form resin was washed on a glass filter with 13 volumes of the 0.25 *N* buffer of pH 7.0, suspended in about 1.5 volumes of the same buffer in a beaker, and pH of the suspension was adjusted to 7.3 with 30 per cent ammonia water. As the pH fell rapidly, ammonia was further added, every time with stirring several minutes to maintain the suspension at pH 7.3. After continuing this procedure for 1 hour the suspension was allowed to stand overnight. During this period the pH of the suspension usually dropped to 7.0–7.1. If it was below 6.9, ammonia was added to bring the pH of the suspension to 7.0–7.2. The resin was transferred to a glass filter and washed by passing about 10 volumes of the buffer slowly.

Purification and Crystallization of Cyt. c

Step 1. Extraction: About 1.7 kg. of fresh beef heart was freed from fat and ligament and passed through a meat mincer. One kg. of the minced muscle was well mixed with

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* In this paper, the concentration of ammonium phosphate buffer is not represented by the concentration of phosphate as usual but by that of the cation, ammonium ion, since the protein on the resin column is affected by the latter (*13*).

0.4 liter of cold 1 N acetic acid and kept at 2–5° for 1 hour. This treatment decomposes the tissue and fascilitated the extraction of cyt. c, preventing it from modification (if the room temperature is high, the mince should be chilled by mixing with 1/30–1/15 amounts of dry ice powder before mixing with the acid). After this treatment, 0.1 liter of 4 N ammonium hydroxide and 400 g. of powdered AS (passed through a 10–20 mesh sieve) were well mixed with the tissue, and the resulting thick suspension was allowed to stand at room temperature (about 20°) for 3 hours or in cold room (about 5°) overnight. The suspension was squeezed out in a press through cloth (1st extract: slightly turbid, reddish brown solution, *ca.* 1.1 liter, 50 per cent saturated AS, pH 6.5). The residue was suspended into 0.5 liter of 50 per cent saturated AS and pressed again after 1 hour (2nd extract: weak brown solution, *ca.* 0.5 liter, pH 6.5).

Step 2. AS Treatment: The combined extracts (*ca.* 1.6 liter, 50 per cent saturated AS, pH 6.5) were adjusted to pH 7.0 with 30 per cent ammonia and solid AS was slowly added to the solution with stirring until the specific gravity became 1.220. After standing at room temperature for 1 hour, the suspension was filtered through a Buchner funnel with the aid of 100 g. of Hyflo Super-Cel, and the residue was washed with about 0.3 liter of 85 per cent saturated AS.

Step 3. TCA-AS Precipitation: The combined filtrate and washing (clear pink solution, *ca.* 1.7 liter, 80 per cent saturated AS pH 6.8) were chilled below 4° and 3 N TCA was added with stirring until the pH of the solution became 4.8 to 5.0 (at this time about 10 ml. portion of the suspension was filtered through a small paper and, if the filtrate still contained cyt. c, a little more TCA was added). After keeping the suspension at temperatures below 4° for 10 minutes, 5 g. of Hyflo Super-Cel, which had been well suspended in 20 ml. of saturated AS, was added with stirring, and then the suspension was filtered as rapidly as possible through a filter paper (*ca.* 15 cm. in diameter) covered with a thin layer of the mixture of paper pulp and celite on a Buchner funnel. The precipitate was washed with 0.1 liter of cold 90 per cent saturated AS and the washings was sucked out. The drained cake was rapidly removed from the paper and immediately suspended in 50 ml. of water. The filter paper, to which a small amount of the cyt. c precipitate still adhered, was also suspended in another 50 ml. of water. Both suspensions were adjusted to pH 7.0 with ammonia and kept for 30 minutes. The former suspension was filtered through a small glass filter, and the remaining celite on the filter was washed with the latter suspension and then with 50 ml. of water. The combined filtrate and washings (*ca.* 140 ml. of clear red solution, pH 7.0) were dialyzed in a cellophane bag against 1 liter of distilled water (dialyzed filtrate: *ca.* 250 ml., pH 7.0, containing NH_4^+ below 0.1 N).

Step 4. Adsorption and Elution: A small resin column was made by transferring the suspension of Am XE-64 equilibrated with 0.25 N buffer of pH 7.0 to Allihn's glass filter, 2.5 cm. in diameter, until the height of the resin bed became about 5 cm., and the bed was washed with 10 ml. of distilled water (see Fig. 2). The dialyzed solution of cyt. c obtained in the foregoing step was oxidized by the addition of a small amount of 0.01 M potassium ferricyanide until the reduced band of cyt. c at 550 mμ could not be observed spectroscopically, then this solution was passed through the above resin bed by gentle suction. The column was washed by passing about 200 ml. of 0.125 N buffer, pH 7.0. The part of the resin colored in red by the adsorption of cyt. c was taken out in the form of the suspension in 0.02 N buffer and transferred to another column, about 1.5 cm. in diameter, then, 0.5 N buffer of pH 7.0 was slowly passed for the elution of the cytochrome (*ca.* 5 ml., pH 6.5–6.8, containing NH_4^+ below 0.5 N) (see Results and Discussion).

Step 5. Chromatography: The eluate, after addition of a few drops of 0.01 M potassium ferricyanide, was diluted with the same volume of distilled water, adjusted to pH 7.0 with ammonia, and then passed through a column of the resin equilibrated with 0.25 N buffer, pH 7.0. The column had been prepared by introducing a thick suspension of the buffered resin in 0.25 N buffer (wet resin 2, buffer 1, by volume) into a tube of 2.5 cm. in diameter, which was held strictly vertically, until the settled resin bed became 20 cm. high. After the concentrated cyt. c was passed through, 0.25 N buffer of pH 7.0 containing about 10^{-2} M potassium ferricyanide was passed at a rate of 20–100 ml. per hour on a fraction collector in a room below 15°. The main effluents which appeared around 3–7 hold-up volume of the resin column were collected (*ca.* 100 ml.) (see Fig. 3).

Step 6. Concentration by Resin Column: The combined main effluents were dialyzed overnight against 5 volumes of distilled water in the cold room and adsorbed on the resin column (1.5×2 cm.) in the same way as Step 5. The dark red resin in the upper part of the column (*ca.* 1 ml.) was transferred to another column of 0.7 cm. in diameter and 5 per cent AS solution of pH 8.0 was slowly passed. A very dark band moved down the column and most of cyt. c was eluted in a volume of less than 1.5 ml. in a small centrifuge tube of known weight.

Step 7. Crystallization and Recrystallization: To each 1 g. of the above concentrated cytochrome solution (1–2 g., containing 4–8 per cent cyt. c) was added a half drop of octanol, 0.40 g. of AS and a half drop of 30 per cent ammonia. After dissolving the salt completely, finely powdered AS were added in small portions using a small rod to dissolve each portion completely until the solution became slightly turbid (the turbidity was very difficult to observe without a strong beam of light due to the strong color of cyt. c). The resulting small amounts of precipitate was centrifuged off at 8,000×*g.* for 5 minutes and the supernatant was transferred to another centrifuge tube. The precipitate in the former tube was washed with 0.5 ml. of saturated AS solution of pH 8.5 and the washing was added to the first supernatant (at this step, the amount of precipitate should be such as contains 1/20–1/10 of the total cyt. c, and this was discarded or recovered).

To the combined supernatant and washing were added a few mg. of ascorbic acid and one drop of 30 per cent ammonia and the solution was kept at room temperature (20–25°). If the solution became turbid within 30 minutes, the solution was allowed to stand with a rubber stopper for a few days at 20–25°. If it had not become turbid, small portions of finely powdered AS were added until the solution became turbid and then allowed to stand. More than half of the cyt. c in the solution was usually crystallized as rosette or leaflet form within 1 or 2 days; at this time the suspension was cooled to −5° and about 1/3 volume of AS solution of pH 9 saturated at 50 per cent was added with stirring. After standing for further several days, the crystals (rosette or fine plates) were collected by centrifugation at 6,000×*g.* and washed with 1 ml. of saturated AS solution of pH 8.5. The washed crystals were dissolved with a minimum volume of water and recrystallized as above after the addition of one drop of 10 per cent ammonia and 1 mg. of ascorbic acid.

RESULTS AND DISCUSSION

Ion Exchange Resin—It was found that two kinds of acrylic polymer having carboxylic group as ion exchange radical, Amberlite IRC-50 and Duolite CS-101, were equally useful for the adsorption and elution procedure (Step 4), chromatography (Step 5) and also concentration of cyt. c (Step 6). The size of the resin particle considerably affected the adsorption capacity as well as the chromatographic behavior of the pigment. Fine particles in Na-form less than 200 mesh were desirable. Thus, powdered Amberlite IRC-50, Am XE-64, was thought to be most convenient for the present purposes.

Both large particles and very fine particles, which made the pass of solution through the resin difficult, should be removed by sieving and decantation.

The resin once used for the treatment of crude cyt. c solution was usually colored in slightly brownish red even if it had been treated with sodium hydroxide, and this resin was conveniently purified to the colorless state by treatment with hypochlorite without loss of the adsorption capacity of cyt. c. Even in the case of fresh resin, this treatment is useful for further purification of the resin. In addition, the treatment of the resin with this oxidizing agent much prevented the reduction of cyt. c during the adsorption and elution process and chromatography. As already reported by other workers (*5*, *6*), a considerable amount of oxidized cyt. c was converted to the reduced form which had a smaller affinity to the resin in the course of the usual chromatography and this phenomenon made the sufficient purification very difficult.

Extraction—Cyt. c had been thought to be stable toward acid and heat treatments on the basis of the fact that the typical adsorption spectrum and catalytic activity of cyt. c are not altered after these treatments. However, as reported recently by some workers (*6*, *9*, *14*), cyt. c can often be modified by these treatments, and the modified pigment can be distinguished from the intact one by means of ion exchange chromatography. Although such modification was reported to be caused by the TCA-AS precipitation or heat treatment, it was thought that drastic treatments should be avoided, if possible, even in those cases when no alteration of the adsorption spectrum of cyt. c could be detected. For these reasons, extraction of cyt. c with concentrated AS solution at neutrality, rather than routinely used TCA extraction, was used in the previous reports (*8*, *9*, *15*). The pigment could be extracted more easily at neutral pH's in the presence of concentrated salt as compared to the extraction with water. This extraction was, however, still more difficult than the acid extraction and, as reported, reextraction was needed.

Further investigations showed that better extraction of cyt. c with acid merely due to destruction of tissue and not to higher solubility of cyt. c at acidic pH's. Moreover, it was found that such destruction of tissue can effectively be achieved with mild acid such as acetic acid in the cold. For the extraction of cyt. c from the destructed tissue, acidity of the solution was not essential but even undesirable in view of higher adsorption of cyt. c on denatured tissue. Based upon the above fact, the muscle was treated with 0.4 volume of 1 *N* acetic acid (final pH about 4.3) in the cold for a limited period of time and extraction of cyt. c was then carried out with 50 per cent saturated AS solution of neutral pH (about 6.0) at room temperature (Step 1). In this method, uneven distribution of acidity in the tissue mixture was sufficiently avoided by the strong bufferring capacity of acetic acid and extraction of impure proteins were greatly prevented by the presence of a high concentration of AS. The squeezing process, therefore, became very easy.

Precipitation of Cyt. c with TCA in AS Solution—The classical Keilin and Hartree's method (*2*) was slightly modified in the present method, in which cyt. c was precipitated at pH 4.5–5 instead of pH 3.7 used by them, since the pigment is completely precipitatable even at this pH if a higher concentration of AS is employed. In fact, if the solution of cyt. c was nearly saturated with AS, the protein was easily precipitated by the addition of TCA even at final pH of 9. It is thought that a complex between cyt. c and TCA is formed at every pH, and precipitation by the TCA-AS method is a kind of salting out of this TCA-cyt. c complex which have lower solubility in AS solution than free cyt. c. The modification of cyt. c accompanying this proceure (*6*, *9*, *14*, *16*) is though to be mainly due to instability of this TCA-cyt. c complex. Though denaturation of proteins usually takes place in their solution and not in solid state, modification of

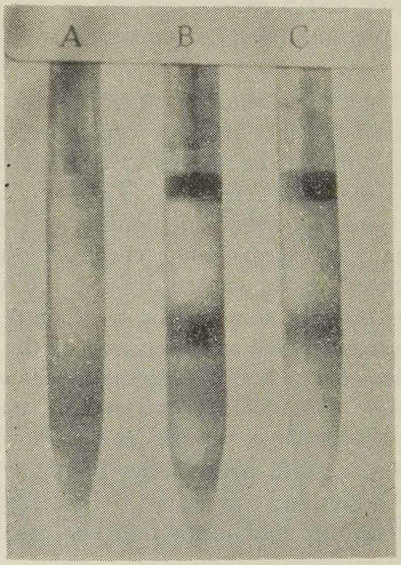


FIG. 1. Chromatograms of cyt. c preparations obtained with and without the TCA-AS precipitation method.

A. The heart extract was dialyzed, directly adsorbed on an Am XE-64 column (1.0×5 cm.), and developed by passing 0.20 *N* NH_4^+ buffer of pH 7.0 (5 hold-up volumes of the resin bed).

B. Cyt. c was precipitated from the extract by the TCA-AS method, immediately dissolved, adsorbed on the column, and then developed as above.

C. Cyt. c precipitated by the same method allowed to stand at room temperature (25°) for 3 hours, and treated as above.

cyt. c was easily caused in the precipitated state of TCA-cyt. c complex. In Fig. 1 are shown chromatograms of several cyt c preparations; one was without the use of TCA-AS procedure, the 2nd by the method in the cold and dissolved immediately, and the 3rd was allowed to stand at room temperature after the precipitation by TCA-AS method. It will be seen that, if the TCA-AS precipitate was kept at room temperature for 3 hours even after the precipitate was washed with AS solution to remove excess TCA, almost all cyt. c was modified.

Since this modification is mainly due to the instability of TCA-cyt. c complex, it can not sufficiently be avoided even if the TCA-AS precipitation is carried out at neutral or alkaline pH. The most important things to be done to prevent the modification is to carry out the procedure as rapidly as possible in the cold and to dissolve the precipitate immediately.

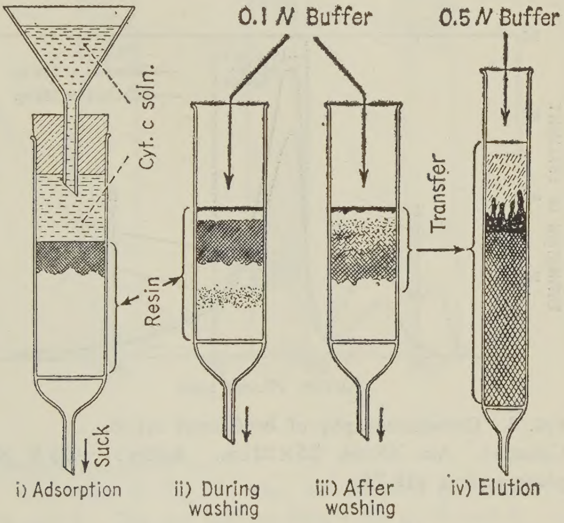


FIG. 2. Procedure in the adsorption and elution process.

Adsorption and Elution Process—The process was conveniently carried out by the method as shown in Fig. 2. If the cyt. c solution charged contained about 0.1 g. ion of NH_4^+ per liter (0.05 M AS) only cyt. c was adsorbed on the column while myoglobin and other proteins passed through. If the solution contained a lower concentration of salt, both cyt. c and myoglobin were adsorbed. When the 0.1 *N* buffer was passed through the column, myoglobin and other proteins were almost completely washed out (Fig. 2, ii), and the band of cyt. c spread (if the charged cyt. c had a cation concentration lower than 0.125 g. ion per liter) and then gradually moved down from the top (Fig. 2, iii). Since this modified cyt. c could be completely separated from the intact pigment by the following chromatography, there was no need to separate this band from the main cyt. c band at this step.

Though cyt. c adsorbed on the washed column can be eluted immediately by passing 0.5 *N* buffer through the column, it is desirable to transfer the resin to another small column after suspending it in dilute buffer in order to elute cyt. c as a very concentrated solution (Fig. 2, iv)

Chromatogrophy—Paléus and Neilands (*5*) used 0.1 *M* ammonium acetate-ammonium hydroxide buffer at alkaline pH for the fractional elution of cyt. c from Amberlite IRC-50. It was elucidated by Boardman and Partridge (*13*) that the affinity of cyt. c to the same resin is smaller as the pH and cationic concentration become higher and that the effect of pH is not pronounced between pH 7 to 8.5. Margoliash (*6*) used 0.25 *M* ammonium acetate, pH 7, for the chromatography. We used ammonium phosphate buffer of pH 7.0 containing 0.25 g. ion of ammonium ion per liter, since this buffer possesses a stronger buffering capacity than ammonium acetate and the resin can be kept strictly under a constant condition. In addition, the resin was equilibrated with buffer before use, in contrast to the previous workers who employed the washed NH_4^+-form resin. In the method of Paléus and Neilands as well as that of Margoliash, partial reduction of cyt. c was observed in the course of chromatography. It was, therefore, impossible for them to obtain all the intact cyt. c as a single fraction, since the reduced cyt. c has much smaller affinity to the resin as compared to the oxidized form. Therefore, it is necessary to carry out the chromatography in such a way that all of the cyt. c is kept in either oxidized or reduced form in order to isolate cyt. c contaminated with least impurities. For this purpose, the resin was treated with hypochlorite to remove any reducing substances before use and a minute amount of ferricyanide was mixed in the development buffer.

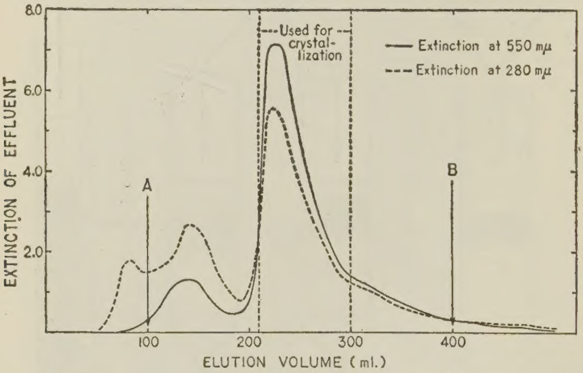


FIG. 3. Chromatography of beef heart cyt. c.
Column: Am XE-64, 2.5×20 cm. Buffer: 0.25 *N* NH_4^+-phosphate system, pH 7.0.

A typical elution curve thus obtained is illustrated in Fig. 3. As can be seen, the main fraction of cyt. c in this particular case emerged in the effluent when about 3 resin-bed volumes of buffer had been passed the column and ended at around 5 resin-bed volumes. The precise position of the appearance of cyt. c into the effluent was considerably affected by the amount of cyt. c charged per unit resin volume and to some extent by the size of resin particules. Before the appearance of the main cyt. c fraction, some colorless proteins and a small amount of myoglobin still contained in the charged solution were usually eluted together with a small amount of cyt. c, which seemed to be modified or in combination with impurities. It was occasionally observed that a small peak (or shoulder) consisting of cyt. c appeared just following the main peak. Though cyt. c in this peak was crystallized in the same shape as that of the main peak, this is thought to be very slightly modified one. As shown in Fig. 4, after the main band of cyt. c had passed the column, usually two bands still remained in the column, one at the top and the other in the middle. The latter band was usually slight. The fairly large band at the top of column was further divided into more than several fractions by passing 0.35 to 0.5 N buffer. These remaining fractions of cyt. c in the column increased if the charged cyt. c preparation had not been prepared carefully. If the main, intact fraction of cyt. c was chromatographed as above after being treated TCA or heat, the main band could no more been observed or considerably decreased and the bands of modified cyt. c appeared instead. Cyt. c in the main band is very resistant to the action of a crystalline bacterial proteinase, while that in the remaining bands is susceptible (*14*). From

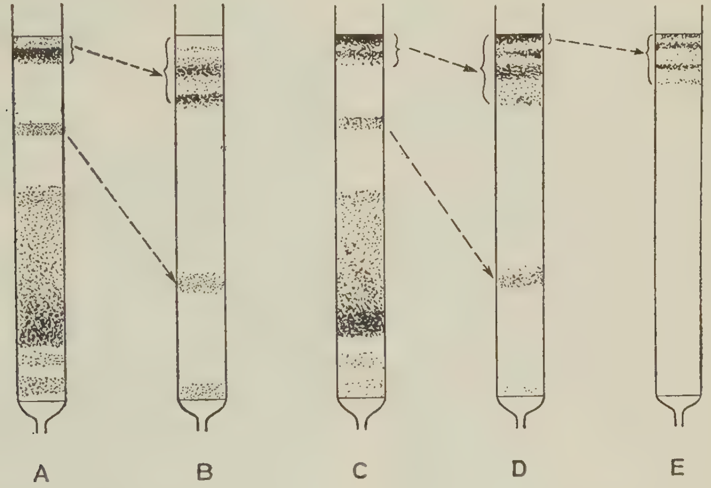
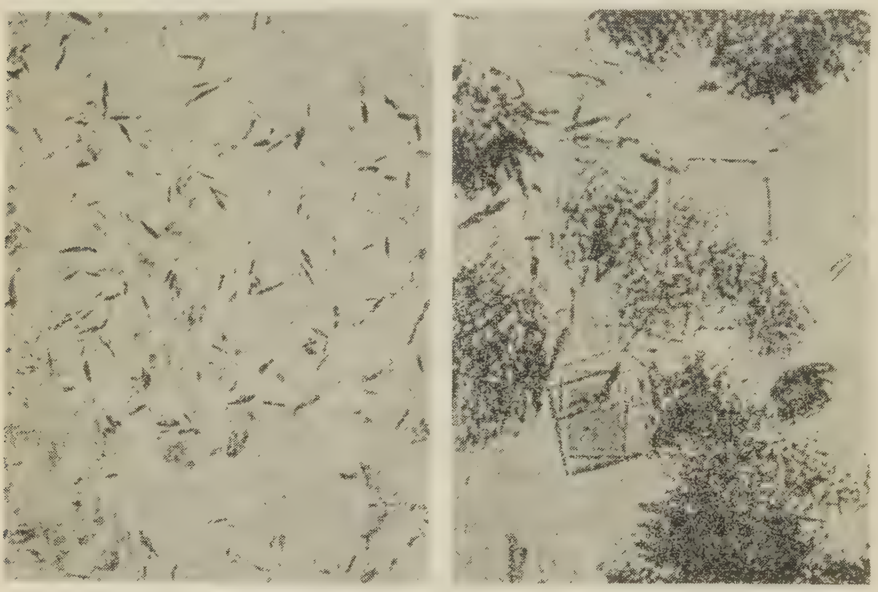


FIG. 4. Chromatograms of cyt. c.

Columns: The same as Fig. 3. Cyt. c: Purified preparation according to Step 1–4 for A and B. The one without Step 4 for C, D and E. Passed buffer: 0.25 N NH_4^+, pH 7.0, 100 ml. for A and C; 0.25 N NH_4^+, pH 7.0, 400 ml. for B and D; 0.50 N NH_4^+, pH 7.0, 100 ml. after the above for E.

these facts, cyt. c in the main band is thought to be intact (or least modified) cyt. c and those in the remaining bands to be modified. The modified preparations of cyt. c usually have higher extinction ratio, R_{280}^{550}, than the intact one; the ratio sometimes exceeding 1.4.

Crystallization of Cyt. c—It was reported by Bodo (*7*) that cyt. c of penguin muscle was crystallized in reduced form in nearly saturated AS solution containing dithionite. Though mammalian cyt. c can be precipitated with AS of impurities or TCA in the solution, the pure pigment is soluble even in saturated AS solution when the concentration is low. Since it had been reported that the reduced cyt. c was less soluble than the oxidized one (*16*), it was thought that the crystallization by AS would be most easily accomplished from a very concentrated solution of the reduced pigment as in the case of the achievement by Bodo. Ascorbic acid was used as reducing agent in the present method instead of dithionite, since the latter seemed to be drastic to cyt. c and might yield crystals of sodium sulfate. Cyt. c was not completely reduced at neutral or acidic pH's, but quite easily at weak alkalinity. The crystallization seemed also to be easier from weak alkaline media (pH 8–9.5) than from neutral solutions.



A. Leaflet form (×400) B. Plate and rosette forms

FIG. 5. Crystalline reduced cytochrome c from beef heart.

When powdered AS was added in small portions to a concentrated reduced cyt. c solution, the part of the solution which became in contact with the added AS usually turned to be turbid at the beginning of the addition, and the turbidity gradually disappeared by gentle stirring. The suitable condition of the solution to yield crystals of cyt. c was that in which the turbidity became persisting. However, the solution sometimes became viscous without forming turbidity by the addition of AS, especially when cyt. c was very concentrated. This is presumably due to the appearance of invisibly small crystals. In either case, the crystals grew fairly rapidly if the solution was allowed to stand at 20–25° (but very slowly in the cold). The crystals usually appeared as rosette or leaflet form rather diluted cyt. c solution (2–4 per cent) and as fine plates from very concentrated solution (see Fig. 5)*. The crystalline material was shown to be quite homogeneous by electrophoretic and ultracentrifugal criteria.

Yield and Purity at Each Step of Purification and Crystallization—A representative result is shown in Table I. The yield of the crystals was 60–90 mg. from 1 kg. of minced heart.

Table I

Summary of Purification Procedure

Steps and treatment	Volume (*ml.*)	Amount of cyt. c		Yield (%)	Purity $\frac{E_{550}\,(\text{red.})}{E_{280}\,(\text{ox.})}$
		(μM/*ml.*)	total (μM)		
1. Extraction	1550				*ca.* 0.03
2. After AS treatment	1700	0.011	18.7	100	*ca.* 0.04
3. TCA-AS precipitation	150	0.11	16.8	89.8	0.85
4. Adsorption and elution	8	1.80	14.4	77.0	1.16
5. Chromatography	120	0.088	10.6	56.7	1.25
6. Concentration	*ca.* 2.0	*ca.* 4.9	*ca.* 9.8	52.4	1.25
7. 1st crystallization	*ca.* 2.5	*ca.* 3.2	*ca.* 8.0	42.8	1.25
2nd crystallization	2.0	3.1	6.2	33.1	1.25

Crystallization of Cyt c from Other Mammalian Muscle—Using the same procedure applied to beef heart, cyt. c was also crystallized from pig and horse hearts and also horse thigh muscle. The crystals obtained from pig heart were similar to those from beef heart but the yield was somewhat lower (see Fig. 6). The yield from horse heart was slightly higher than that from beef heart, but the crystals were very fine needles (or plates). The crystals from horse thigh muscle was quite the same as those from horse in their appearance, but the yield from the same amount of muscle was several times lower. In the above three cases, the purity and relative yield of cyt. c at each step of purification and crystallization were almost the same as in the case of beef heart.

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* The shape of unit crystal was once thought to be needly at first (*8*), but it must be very thin square plate since the needle-like crystals were often converted into the latter by the long standing of the suspension of crystals or by recrystallization from a very concentrated solution.

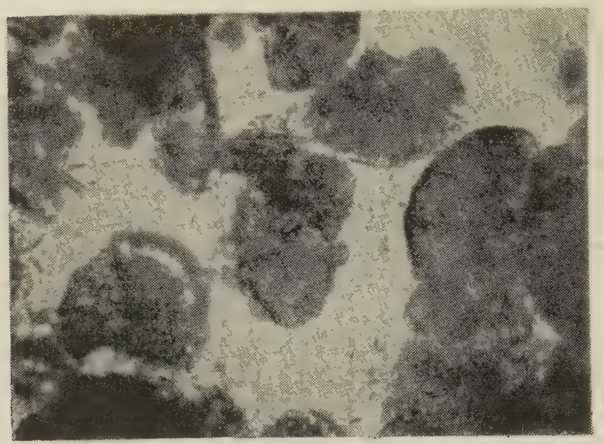

FIG. 6. Crystalline reduced cytochrome c from pig heart. (Rosette, ×400)

SUMMARY

Crystalline cytochrome c was prepared as follows; cyt. c was extracted with concentrated solution of ammonium sulfate from beef heart after treatment with acetic acid. After removal of the other proteins with ammonium sulfate, cyt. c was precipitated with trichloroacetic acid, and purified by adsorption and elution on a carboxylic acid cation exchanger. The purified protein was chromatographed on the same resin column equilibrated with ammonium phosphate buffer, pH 7, containing a small amount of ferricyanide, and cyt. c in the main fraction was concentrated by a resin column and crystallized from ammonium sulfate solution after reduction with ascorbic acid. The method was also applicable for the crystallization of cyt. c from other mammalian muscle such as pig heart, horse heart and thigh muscle.

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CRYSTALLINE CYTOCHROME C

II. CRYSTALLIZATION OF PIGEON CYTOCHROME C AND A COMPARISON OF TWO CRYSTALLIZATION METHODS

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It was reported in our previous papers (*1–3*) that mammalian cytochrome c can be crystallized from beef, pig and horse hearts and horse thigh muscle. The cytochrome pigment of avian origin, on the other hand, was first purified by Paléus (*4*) from chicken heart and, later, Bodo (*5*) reported on its crystallization from penguin muscle using the TCA-AS** precipitation and resin adsorption method.

We have also succeeded in crystallizing the pigment from pigeon breast muscle (*6*) by a TCA-AS method similar to that applied for mammalian hearts (*3*). Pigeon breast muscle was chosen as the avian source since this is known to contain the highest concentration of cyt. c among a variety of tissues so far examined.

It was, however, noticed that in the isolation procedure employed a considerable portion of the pigment undergoes modification even if it is carefully and rapidly performed in the cold. In the present investigation, therefore, we have introduced a new isolation method (Method II) instead of the previous one (Method I) and thus succeeded in greatly, if not completely, preventing the modification of the pigment and attaining a substantial increase in the yield of crystals. The new method in which the use of TCA is avoided is characterized by the adsorption of cyt. c on the resin Amberlite XE-64 at weakly acidic pH values.

The purpose of the present paper is to describe the details of the new method and to compare it with the older one. The pigeon pigment could be crystallized not only in the reduced form as that of mammalian tissues but also in the oxidized form.

EXPERIMENTAL

Assay Methods

Cyt. c was assayed by the same methods as employed in the previous report (*3*).

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** The following abbreviation are used in this paper: TCA for trichloroacetic acid, AS for ammonium sulfate, cyt. c for cytochrome c, and Am. XE-64 for Amberlite XE-64.

Preparation of Resin

The ion-exchange resin Amberlite XE-64 was passed through a 200 mesh sieve, suspended in water, and freed from finer particles by repeated decantation each after settling for 3 hours. It was collected and treated with sodium hydroxide, hypochlorite and hydrochloric acid as previously described (*3*). The H-form resin thus obtained was further treated in two different ways for different purposes. (*a*) The resin was washed with 50 per cent saturated AS, suspended in the same AS solution, and then adjusted to pH 4.5–4.7 with 30 per cent aqueous ammonia (resin A). (*b*) The resin was equilibrated with 0.25 N (0.25 g. ion of NH_4^+) ammonium phosphate buffer of pH 7.0 as described in the previous paper (*3*) (resin B).

Purification and Crystallization of Cyt. c

Method I

This was essentially the same as employed in the previous report (*3*) for the purification and crystallization of cyt. c from beef heart.

Method II

Step 1. Extraction—The extraction procedure used in Method I (*3*) was slightly modified. One kg. of minced pigeon muscle was treated with 0.4 liter of 1 N acetic acid as previously described (*3*), and then 450 g. of powdered AS were dissolved in the acidic suspension (about pH 4.3). After standing for a few minutes, the pH of the mixture was adjusted to 4.5–4.7 with 30 per cent ammonia, and then allowed to stand for 2 hours at 10–15° or overnight below 2°. The suspension was squeezed in a press, and the residue was reextracted for 1 hour with 0.5 liter of 55 per cent saturated AS solution of pH 4.5. It was squeezed again. The combined extracts were chilled (below 10°) and filtered through a Buchner funnel with the aid of 50 g. of Hyflo Super-Cel and the residue was washed with a small amount of the AS solution (combined filtrates, *ca.* 1.6 liter).

Step 2. AS Treatment—The pH of the combined filtrates was adjusted to 6.5–7.0 and treated with AS in the same way as described for Step 2 in Method I (*3*).

Step 3. Absorption on Resin and Elution—The combined filtrates and washing from Step 2 (clear pink solution, *ca.* 1.7 liter, pH 6.7, *ca.* 80 per cent saturated with respect to AS) were diluted* with a half volume of distilled water (*ca.* 2.5 liters, *ca.* 55 per cent saturated with respect to AS), chillied to 5° or lower, adjusted to pH 4.5–4.7 with glacial acetic acid, and rapidly passed by suction through a bed of resin A contained in a glass filter (10 cm. in diameter and 8 cm. in height).

The resin bed, colored in pale red, was washed with 1.2 liter of 0.05 N ammonium phosphate buffer of pH 7.0, transferred into a beaker, and suspended in 1 liter of cold 0.05 N buffer**. The suspension*** was adjusted to pH 7.5 by the addition of 30 per cent ammonia with stirring (chilled in ice water), and poured into a glass filter in which a 1 cm. layer of fresh resin B had already been placed (see Fig. 1). The whole resin in the glass filter was washed with 0.125 N buffer**** of pH 7.0 until a minute amount of cyt. c began to be eluted out. This usually required 2–5 liters of the buffer. The washed bed was then slowly eluted with 1 N buffer of pH 7.0 and the reddish eluate was collected (*ca.*

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* Dilution was made in order to avoid the precipitation of impurities accompanying the lowering of pH to 4.5–4.7.

** If a large amount of the resin at the lower part of the bed was still colorless, this part should be carefully removed.

*** Most of the resin was still in the H-form.

**** 0.1 N buffer for mammalian cyt. c.

150 ml., *ca.* pH 6.5, *ca.* 0.8 N with respect to NH_4^+).

Step 4. Second Adsorption and Elution—The eluate obtained from Step 3 was diluted with 4 volumes of distilled water, adjusted to pH 7.0 with a few drops of 30 per cent ammonia, and 0.01 M potassium ferricyanide was added in order to oxidize cyt. c completely. The oxidized solution (*ca.* 800 ml., 0.15 N with respect to NH_4^+) was passed through a bed of resin B (4 cm. in diameter and 8 cm. in height) placed in a glass filter and the resin was washed with 0.175 N buffer* of pH 7.0, until the colored band of cyt. c moved down about 2 cm. Then the colored part of resin was carefully taken out and suspended in 0.02 N buffer. The suspension was transferred to a column of 2 cm. in diameter and cyt. c was eluted with 0.5 N buffer in the same way as described for Step 4 in Method I (*3*) (eluate, *ca.* 10 ml., less than 0.5 N with respect to NH_4^+).

Step 5 Chromatography, Step 6. Concentration by Resin Column, and *Step 7. Crystallization of Reduced Cyt. c*—These three steps were carried out in the same way as described for Steps 5, 6 and 7, respectively, in Method I (*3*)

Crystallization of Cyt. c in the Oxidized Form

The concentrated solution of cyt. c (3–6 per cent) obtained from Step 6 of the above method was adjusted to pH 7.0–8.5 by the addition of one drop of 30 per cent ammonia per 1 g. of solution and treated with 0.45 g. of AS per 1 g. of solution in a similar manner to that for the crystallization of reduced cyt. c (*3*). The precipitate, if any, was removed by centrifugation and powdered AS was carefully added at room temperature (15–20°) using a small glass rod to dissolve each portion of AS completely until the solution became slightly turbid. When the slightly turbid solution was kept at room temperature, an amorphous precipitate of cyt. c appeared at first, but in 1–2 weeks crystals of the oxidized pigment were gradually formed in needles or slant plates. The amorphous precipitate disappeared gradually as the crystallization proceeded.

DISCUSSION

Extraction of Cyt. c—In both Methods I and II cyt. c is extracted in the cold from acetic acid treated mince with about 55 per cent saturated AS solution. The only difference is that the extraction is performed at neutral pH's in the former method while at about pH 4.5–4.7 in the latter. The amount of cyt. c extractable from the acid-treated mince with the AS solution is not affected by the pH of solution in the range of pH 4.5 to 8.0, although more cyt. c can be extracted from intact tissues at acidic than at neutral pH's. It was, however, found that much larger amounts of impure proteins were extracted at neutral pH's than at acidic pH's, a fact which makes the adsorption of cyt. c on the resin quite difficult. In Method II, therefore, acidic pH's are prefered for the extraction procedure. Little cyt. c undergoes modifications during the extraction at pH 4.5–4.7 provided the procedure is carried out for a short time at a temperature below 15° or even for a relatively long time at 2°.

AS Treatment—In Method I the AS treatment is carried out not only to remove contaminant proteins but also to raise the AS concentration to such a level that cyt. c becomes easily precipitable by TCA. In Method II in which the use of TCA is avoided, however, the purpose of the treatment consists only in the removal of impure proteins. The resin Am. XE-64 as well as Duolite CS-101 can adsorb, besides cyt. c, only a few contaminant proteins at neutral pH's, but is capable of adsorbing a great many proteins and even non-protein substances at acidic pH's. A large amount of the resin is, therefore, required in this step for the complete adsorption of cyt. c.

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* 0.15 N buffer for mammalian and fish heart cyt. c.

Adsorption of Cyt. c from Solution Containing High Concentrations of Salt—For the effective adsorption of cyt. c from neutral AS solutions on the carboxylic acid resin such as Amberlite IRC-50 (XE-64) or Duolite CS-101, it is necessary to reduce the concentrations of cations (*e.g.*, NH_4^+ and Na^+) to at least 0.2 g. ion by means of dialysis, which is quite troublesome and even impractical for such large amounts of solution. It was, however, found that cyt. c can be adsorbed on the H-form resin even from nearly saturated AS solutions if the pH of the solution is lowered below 5. It carried out in the cold, such a adsorption procedure at acidic pH's causes little modification of cyt. c from both mammalian and pigeon sources. The affinity of the pigment to the resin is, however, much less at acidic pH's as compared to that at neutral pH's, and, as already mentioned above, the resin may adsorb a number of impurities under these conditions. Larger amounts of the resin are, therefore, required in these cases. In addition, the resin should also be washed throughly to remove large amounts of impurities adsorbed.

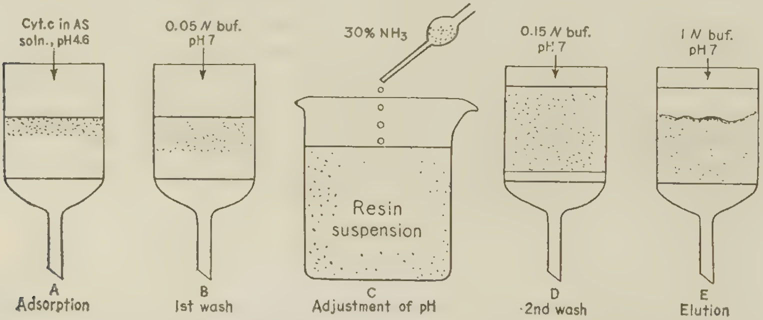

FIG. 1. Process of "Adsorption on Acidic Resin and Elution (Step 3)"

In conclusion, the adsorption can be conveniently carried out as shown in Fig. 1. When the resin bed adsorbed cyt. c from acidic AS solutions is washed with 0.05 *N* buffer of pH 7.0 (Fig. 1, B), a greenish yellow pigment is eluted out, but no other proteins are eluted in this case since most of the resin was still in the acid form due to its strong buffering capacity. The purpose of this procedure is merely to remove the AS and not to wash out other proteins. Even if the resin bed is washed with more than 20 liters of the buffer, it will be impossible to convert all the resin to the neutral form from which other proteins can be eluted. In order to convert the resin from the acid to neutral form without eluting cyt. c, it is necessary to suspend the wash resin in 0.05 *N* buffer and to adjust the pH of the suspension to 7.5 with concentrated ammonia (Fig. 1, C). If the neutralized resin is transferred into a glass filter and 0.05 *N* buffer is passed through it, considerable amounts of impure proteins can be eluted leaving cyt. c on the resin. Impurities can, however, not sufficienty be washed out with this buffer so that cyt. c of more than 30 per cent purity can be obtained. The washing is only sufficient when 0.1-0.15 liter buffer of pH 7.0 is used. With this buffer even a small amount of cyt. c is eluted from the resin. In order to prevent the loss of the pigment in such a washing process, a thin layer of freshly neutralized resin is placed under the resin on which cyt. c is adsorbed (Fig. 1, D). The affinity of pigeon cyt. c to the resin is somewhat different from that of mammalian or fish cyt. c. Therefore, a buffer containing 0.125 g. ion of cation is suitable for the former whereas that containing 0.1 g. ion for the latter. When 1 *N* buffer is slowly passed through the column

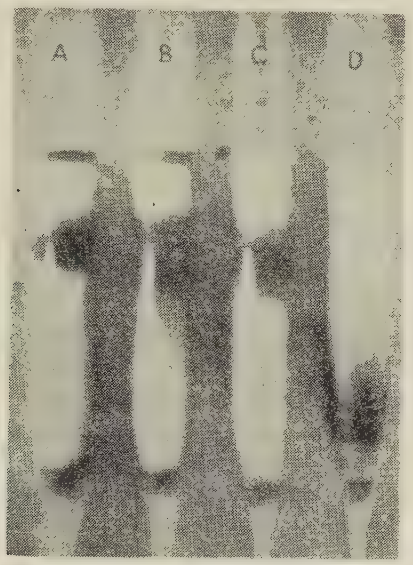

FIG. 2. Comparison of chromatograms of cyt. c obtained by TCA-AS method and the acidic resin adsorption.

A. Pigeon cyt. c precipitated by the TCA-AS method was allowed to stand at room temperature (25°) for 2 hours, and chromatographed on the column (1.0×5 cm.) by passing 0.2 *N* buffer, pH 7.0 corresponding to 5-fold volume of the resin bed (*ca.* 20 ml.).

B. Pigeon cyt. c was precipitated from the extract by the TCA-AS method, immediately dissolved, and then chromatographed as above.

C. Pigeon cyt. c was concentrated by the "Adsorption on Acidic Resin and Elution (Step 3)" and chromatographed as above.

D. Normal fraction of beef heart cyt. c was chromatographed as above.

after the above washing, the dark red band of cyt. c immediately starts to move down from the top of the resin bed (Fig. 1, E). Thus, more than 95 per cent of the adsorbed pigment can easily be recovered in a eluate which is less than 200 ml. The purity of cyt. c in this stage is so high that the extinction ratio (E_{550}/E_{280}) of the solution is in the order of 0.5 to 0.7. As illustrated in Fig. 2, cyt. c's from pigeon breast muscle and beef heart are scarcely modified in the course of the present procedure. Fish heart cyt. c, on the other hand, suffers from modification to some extent unless the procedure is carried out rapidly in the cold (see the following paper). But it should be noted that even in the case of fish heart, the amount of cyt. c modified during the present procedure is much smaller than during the TCA-AS precipitation method.

Second Adsorption and Elution—It appears that the crystallization of cyt. c is greatly prevented by the presence of impurities whose affinities to the resin are slightly less than that of cyt. c. If most of such impurities are not removed, considerable amounts of them are usually contaminated in the main fraction of cyt. c in the subsequent chromatography and render the crystallization very difficult. On the contrary, the other impure proteins possessing higher affinities can be easily separated from the main cyt. c fraction by a single chromatography. In Step 4 Method II, therefore, the resin bed on which cyt. c is adsorbed is throughly washed with 0.175 *N* (for pigeon) or 0.15 *N* (for mammals and fish) buffer* until the band of cyt. c moves down a few cm. Such a careful washing is not necessary for cyt. c preparations purified by the TCA-AS precipitation method (*3*). In this washing process, the cyt. c adsorbed on the resin bed should be in the oxidized form, since the affinity of reduced cyt. c is considerably less than that of oxidized one. Even if all the cyt. c is in the oxidized form, a small portion of the pigment may be eluted out, although the main band of cyt. c is still remaining on the resin. This portion may have undergone modifications or formed a complex with some impurity.

Chromatography—For the sufficient purification of pigeon breast muscle cyt. c by ion-exchange chromatography, it seems that buffer solutions of pH 7.0 containing 0.225–0.30 g. ion of cation (NH_4^+) are suitable. For mammalian and fish cyt. c, on the other hand, buffer solutions containing 0.2–0.275 g. ion of NH_4^+ are useful. If a buffer of lower concentration is used, the band of the cyt. c becomes narrower and a smaller column can be employed. In such a case, however, cyt. c in the main effluent will be greatly diluted due to the slower movement. For the sake of convenience, buffer containing 0.25 g. ion of NH_4^+ is used for chromatography of both pigeon and mammalian cyt. c in the proposed method. Although the amount of cyt. c extracted from pigeon breast muscle is fairly higher than that from the same weight of beef heart, almost the same size of the resin column is applicablbe for the both cases because of the higher affinity of pigeon cyt.

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* The pigeon cyt. c possesses a higher affinity to the resin as compared to those of mammalian and fish ones.

c. Typical chromatograms of pigeon breast muscle and beef heart cyt. c obtained by this means are shown in Fig. 3 and 4, respectively. As can be seen from the figures, some impure proteins and a small amount of cyt. c of lower purity still appears before the main effluent of the pigment, even though most of the impurities possessing less affinity have already been washed out in the preceeding step. When the main band of cyt. c has been eluted out, no remarkable colored material remains in the column in the case of Method II if Step 3 has been carried out rapidly in the cold. In Method I which includes the TCA-AS precipitation procedure, however, a few bands of the modified pigment still remain in the column at this stage. In both Methods I and II, the extinction ratio E_{550}/E_{280} of cyt. c in the main effluent obtained in this step is 1.16±0.02 for pigeon muscle and 1.26±0.02 for beef heart. These values can scarcely be increased by crystallization.

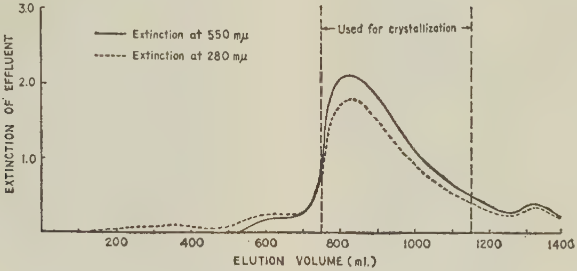


FIG. 3. Chromatography of pigeon breast muscle cyt. c. Column: Amberlite XE-64, 3×20 cm., Buffer: 0.25 N ammonium phosphate (0.25 g. ion of NH_4^+) of pH 7.0, cyt. c preparation: Method II, Step 4.

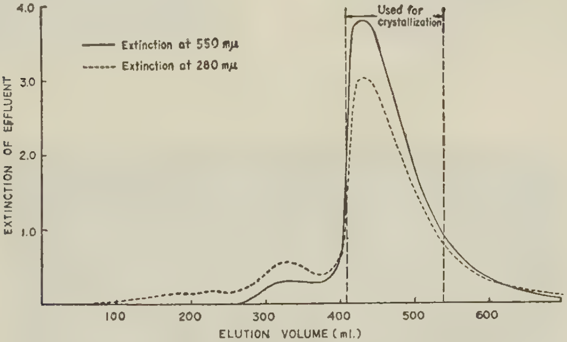
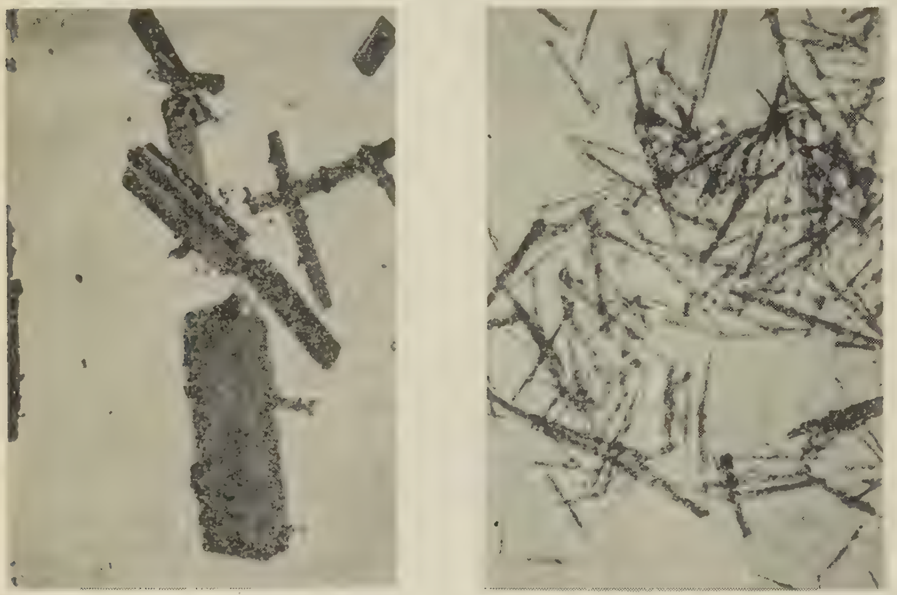


FIG. 4. Chromatography of beef heart cyt. c. Experimental conditions: The same as Fig. 3.

Crystallization of Pigeon Cyt. c—The reduced form of pigeon breast muscle cyt. c can easily be crystallized in a similar way as described for beef heart cyt. c (*3*). The crystals obtained are long plates or needles as shown in

Fig. 5 A and B, respectively, while reduced cyt. c from beef heart crystallized as very fine plates which often group into rosette or leaflet form. The oxidized form of the pigeon pigment can be crystallized as plates or prisms



A. Plate form (×300) B. Needle form (×300)

FIG. 5. Crystalline reduced cytochrome c of pigeon breast muscle.

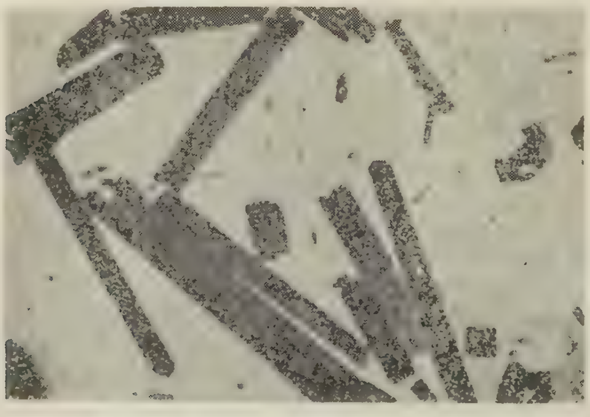


FIG. 6. Crystalline oxidized cytochrome c of pigeon breast muscle. (Prism form, ×300)

(Fig. 6) at lower pH's (6.8) than in the case of the crystallization of reduced cyt. c. As will be reported in the following paper (7), cyt. c's of fish and beef hearts can also be crystallized in the oxidized form.

Comparison of Methods I and II—The yield of recrystallized pigeon cyt. c is 100-150 mg. and 120-180 mg. per kg. of the trimed muscle according to Method I and II, respectively. The lower yield in Method I is mainly due to the modification of the pigment in the TCA-AS precipitation procedure. Method II can also successfully be used for the crystallization of beef heart cyt. c, and the yield per kg. of the material is 80-140 mg., while that by Method I 60-90 mg. (*3*). In Tables I and II are summarized the yield and purity of cyt. c at each step in the two methods from pigeon breast muscle and beef heart. Although Method I usually gives lower yields of the crystals than Method II, this method, if cares are taken to prevent the modification

TABLE I

Summary of Purification and Crystallization of Cyt. c from Pigeon Breast Muscle and Beef Heart by Method II

Steps	from Pigeon muscle			from Beef heart		
	Volume (*ml.*)	Yield* (μM)	Purity (E_{550}/E_{280})	Volume (*ml.*)	Yield* (μM)	Purity (E_{550}/E_{280})
1. Extracts of 1 kg. mince	1600		*ca.* 0.04	1500		*ca.* 0.03
2. After AS treatment	1750	35.0	0.05	1700	17.0	0.04
3. Acidic resin adsorption and elution	180	32.0	0.85	170	14.0	0.80
4. 2nd Adsorption and elution	25	29.0	1.12	22	12.5	1.17
5. Chromatography	380	24.5	1.16	210	11.0	1.28
6. Concentration	*ca.* 4.0		1.16	*ca.* 2.0		1.30
7. 1st crystallization	5.0	20.5	1.16	2.5	8.5	1.28
2nd crystallization	4.0	17.7	1.16	2.0	7.0	1.30

* Total amount of cyt. c is represented by number of micro mole.

TABLE II

Summary of Purification and Crystallizationof Cyt. c from Pigeon Breast Muscle and Beef Heart by Method I

Steps	from Pigeon muscle			from Beef heart		
	Volume (*ml.*)	Yield* (μM)	Purity (E_{550}/E_{280})	Volume (*ml.*)	Yield* (μM)	Purity (E_{550}/E_{280})
1. Extracts of 1 kg. mince	1500			1550		
2. After AS treatment	1700	33.0	0.055	1700	18.0	0.045
3. TCA-AS method	130	31.0	0.95	120	15.0	1.00
4. Adsorption and elution	15	26.5	1.10	12	13.0	1.15
5. Chromatography	340	18.5	1.17	180	9.5	1.27
6. Concentration	*ca.* 3.5		1.15	*ca.* 1.5		1.25
7. 1st crystallization	4.5	16.5	1.19	2.0	7.0	1.30
2nd crystallization	4.0	12.5	1.20	2.0	5.5	1.28

of the pigment, is still a very useful method for the crystallization of animal cyt. c, since the TCA-AS precipitation method (Method I, Step 3) can be carried out more easily and rapidly than the acidic resin adsorption method (Method II, Step 3). The subsequent adsorption and elution process (Step 4) is also easier in Method I than in Method II because of the sufficient removal of impurities by the TCA treatment.

SUMMARY

Details of a new method (Method II) has been presented for the purification and crystallization of cyt. c from animal muscle. In this method cyt. c is directly adsorbed on Amberlite XE-64 at weakly acidic pH's from the extract containing a high concentration of ammonium sulfate and eluted with a buffer instead of the precipitation of the pigment by TCA (Method I). This new method permits the crystallization of cyt. c from pigeon breast muscle in a yield of 120–180 mg. per kg. muscle. This is higher than the figures of 100–150 mg. attainable by Method I. Pigeon cyt. c has also been crystallized in the oxidized form as well as in the reduced form. Method II is also useful for the crystallization of beef heart cyt. c.

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DEHYDROGENATION PRODUCTS OF CONIFERYL ALCOHOL FORMED BY THE ACTION OF MUSHROOM PHENOL OXIDASE, RHUS-LACCASE AND RADISH PEROXIDASE

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(Received for publication. November 19, 1957)

In previous papers one of the authors (T. H.) has reported that Ereudenberg's phenol dehydrogenase, by which a lignin like product is formed from coniferyl alcohol, is identical with laccase contained in crude mushroom extracts. Distribution of laccase in higher plants is, however, very limited, and therefore it is difficult to consider that laccase is responsible for the lignin biosynthesis in common higher plants.

The authors observed that coniferyl alcohol was oxidized in a system composed of D-amino acid apo-oxidase, flavin adenine dinucleotide, D,L-alanine and peroxidase**. In this case, the amount of oxygen consumption by coniferyl alcohol was similar to that observed in the case of laccase, and the dehydrogenation product was proved to have very similar chemical nature to that of coniferous lignin.

Considering the wide distribution of peroxidase in higher plants, it should be suggested that peroxidase may play a main role in lignin biosynthesis (*1*, *2*). The same opinion has been proposed by Lyr recently (*3*).

In the present paper the similarity in the chemical nature of the polymeric dehydrogenation products (DHPs) of coniferyl alcohol obtained by the action of each of mushroom phenol oxidase, Rhus-laccase and radish peroxidase will be reported, and the results obtained from nitrobenzene oxidation, ethanolysis and hydrolysis of the DHPs will be discussed with reference to coniferous lignin.

MATERIALS AND METHODS

Preparation of mushroon phenol oxidase and laccase of Japanese lacquer have been described in the previous paper (*2*), and radish peroxidase was prepared following Kenten and Mann's procedure (*4*).

Coniferyl alcohol (1 g. each) was dissolved in 2.5 liters of phosphate buffer (pH 6.8) and enzyme solution (10 ml. each) was added. Hydrogen peroxide (0.5 per cent total amounts 10 ml.) was added to the mixture at suitable intervals in the experiment with

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** The experimental results will be reported in detail elsewhere.

peroxidase. Each mixture was aerated at room temperature. After 24 hours further 10 ml. of the enzyme was added and aerated for 24 hours; also hydrogen peroxide (10 ml.) was added to the peroxidase mixture at this stage.

All of the reaction mixtures became milky in appearance within several minutes and pale brownish flocculent precipitates were produced gradually. After 48 hours of incubation, the precipitates were collected by centrifugation and dissolved in acetone. The DHPs in acetone were purified by precipitating with benzene (*5*); yield, adout 0.5 g. each. All of the purified DPHs were amorphus powder, and pale brownish in color and gave a strong phloroglucinol-HCl reaction. Methoxyl content was determied by the method of Vieböck (*6*), and found to be 16.4–16.7 per cent.

Nitrobenzene Oxidation—The DHPs (100 mg. each) were autoclaved at 160° in a sealed tube with 2 *N* NaOH (1 ml.) and nitrobenzene (0.1 ml.) for 2.5 hours. After the digests were neutralized, they were throughly extracted with ether. The ether extracts were dried over anhydrous sodium sulfate, and solvents were evaporated. The residue was dissolved in ethanol and used for paper chromatography. Ascending two dimensional paper chromatography was mainly employed using distilled water and *n*-butanol saturated with 3 per cent NH_4OH as solvents. After spraying with diazotized sulfanilic acid and 2,4-dinitrophenylhydrazine-HCl, respectively, the R_f values and color reactions were compared with those of authentic specimens.

Ethanolysis—The DHPs (100 mg. each) were heated at 100° in a sealed tube with 99 per cent ethanol containing 2 per cent hydrogen chloride (1 ml.). After 48 hours the solvents were evaporated under reduced pressure and the residue was extracted completely with petroleum ether. The identification of the petroleum ether-soluble products were made by paper chromatography. The same solvent and spraying reagents as employed in the nitrobenzene oxidation were mainly used, and the R_f values were compared with those of syntetic samples. The method of synthesis of the reference compounds has been described in detail in the previous paper (*7*).

Hydrolysis—The DHPs (100 mg. each) were heated in a sealed tube with distilled water (1 ml.) at 175° for 1 hours (*8*). The reaction mixtures were extracted exhaustively with ether, respectively, and the ether-soluble products were analyzed by paper chromatography. The same solvents and spraying reagents as described in the preceeding sections were used, and a specific reddish purple coloration with phloroglucinol-HCl was utilized for the detection of coniferyl aldehyde.

RESULTS AND DISCUSSION

All of the DHPs obtained were lignin-like amorphous powder, and as showon in Table I gave a strong phloroglucinol-HCl reaction and contained 16.4–16.7 per cent of methoxyl.

It has been well known that vanillin is produced by nitrobenzene oxidation of coniferous lignins. In the present experiment it was shown that the oxidation products of the DHPs contained large quantities of vanillin, which was detected on the paper chromatograms as distinct dark orange spot by spraying 2,4-dinitrophenylhydrazine-HCl. On the other hand, recent study of Kratzl and Schweers proved that Freudenberg's DHP of coniferyl alcohol produced well-known Hibbert's monomers upon ethanolysis (*9*). It was shown in Table II that the DHPs obtained in the present investigation gave also Hibbert's monomers such as 1-ethoxy-1-

TABLE I A

Dehydrogenation Products Obtained by the Action of Phenol Oxidases and Peroxidase

	OCH_3 (%)	Phloroglucinol-HCl reaction
DHP (mushroom phenol oxidase)	16.6	−
DHP (Rhus-laccase)	16.7	+
DHP (radish peroxidase)	16.4	++

TABLE I B

Compounds Obtained by Nitrobenzene Oxidation of DHPs

Compound	R_f value		Color reaction	
			Diazotized sulfanilic acid	2,4-Dinitrophenyl-hydrazine-HCl
Vanillin	0.38*	0.65**	Orange	Orange
Vanillin acid	0.07	0.81	Orange	Colorless

* n-Butanol saturated with 3 per cent NH_4OH.

** Distilled water.

TABLE II

R_f Values and Color Reactions of Compounds Obtained by Ethanolysis of DHPs

Compound	R_f value		Color reaction	
			Diazotized sulfanilic acid	2,4-Dinitrophenyl-hydrazine-HCl
Vanillin	0.38*	0.65**	Orange	Orange
Guaiacyl acetone	0.83	0.77	Reddish orange	Yellow
Vanilloyl methyl ketone	0.54	0.70	Yellow	Yellow
1-Ethoxy-1-(4-hydroxy-3-methoxy-phenyl)-2-propanone	0.90	0.90	Reddish pink	Yellow
2-Ethoxy-1-(4-hydroxy-3-methoxy-phenyl)-1-propanone	0.85	0.87	Reddish orange	Yellow

* n-Butanol saturaed with 3 per cent NH_4OH.

** Distilled water.

(4-hydroxy-3-methoxyphenyl)-2-propanone, 2-ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, guaiacyl acetone, vanilloyl methyl ketone and vanillin by ethanolysis. These products gave yellow to dark orange spots with 2,4-dinitrophenylhydrazine-HCl and yellow to reddish pink spots with diazotized sulfanilic acid, and the R_f values were in good agreement with those of authentic specimens. Of these spots, the one corresponding to 2-

ethoxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone was very distinct.

Goldschmid confirmed recently that coniferyl aldehyde, *p*-hydroxycinnamic aldehyde, vanillin, vanilloyl methyl ketone and guaiacyl acetone were formed by the hydrolysis of "extractive-free western hemlock wood and Brauns' hemlock native lignin (*8*). As shown in Table III, four compounds giving distinct colored spots corresponding to coniferyl aldehyde, vanillin, vanilloyl methyl ketone and guaiacyl acetone respectively were detected from the hydrolysis products of the DHPs. Of these compounds, coniferyl aldehyde was identified by means of its R_f value and by a specific reddish purple coloration with phloroglucinol-HCl.

TABLE III

R_f Values and Color Reactions of Compounds Obtained by Hydrolysis of DHPs

Compound	R_f value		Color reaction	
			Diazotized sulfanilic acid	2,4-Dinitrophenylhydrazine-HCl
Vanllin	0.38*	0.65**	Orange	Orange
Guaiacyl acetone	0.83	0.77	Reddish orange	Yellow
Vanilloyl methyl ketone	0.54	0.70	Yellow	Yellow
Coniferyl aldehyde	0.45	0.44	Reddish pink	Yellow

* *n*-Butanol saturated with 3 per cent NH_4OH.

** Distilled water

The results of the above mentioned experiments indicate that a close resemblance exists in the chemical nature of the DHPs of coniferyl alcohol obtained by the action of mushroom phenol oxidase, Rhus-laccase and radish peroxidase, and that these DHPs are very similar to native coniferous lignin. In view of the wide distribution of peroxidase in higher plants these findings seem to favor the author's suggestion that the enzyme responsible for the biosynthesis of lignin from coniferyl alcohol might probably be peroxidase.

SUMMARY

The DHPs of coniferyl alcohol were obtained by the action of each of mushroom phenol oxidase, Rhus-laccase and radish peroxidase. These DHPs gave a positive phloroglucinol-HCl reaction and contained 16.4–16.7 per cent of methoxyl. The DHPs obtained by the action of these different enzymes are similar in chemical nature, and produced the same degradation products as obtained from coniferous lignin by nitrobenzene oxidation, ethanolysis and hydrolysis.

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STUDIES ON THE PROTEIN METABOLISM OF REGENERATING LIVER (I)*

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Starting with the work of Schoenheimer (*1*), there have been quite many attempts to approach the problem of the biosynthesis of protein, using a suitable isotope-labeled amino acids. Most workers in this field of study in the past, however, investigated the incorporation of labeled amino acids only into the whole, unfractionated tissue protein, *i.e.*, a mixture of various kinds of protein of the tissues as a whole, and until recently, a relatively few reports have been published concerning the incorporation of labeled amino acids into purified fractions of the animal tissue protein. Especially, little is known about the metabolism of the protein moieties of nucleoprotein, although there have been many investigations concerning that of nucleic acid moieties. Brunish and Luck (*2*), in their *in vivo* studies using C^{14}-labeled phenylalanine and alanine, observed the incorporation of these amino acids into desoxyribonucleohistone, histone, and soluble protein fraction of the nuclei of rat liver cells. They reported that the histone appeared to be less active metabolically than the other proteins examined. Littlefield *et al.* (*3*), using cell-free system, observed that the ribonucleoprotein particles obtained from the Ehrlich mouse ascites tumors take up C^{14}-amino acids more rapidly than the soluble proteins of the tumor cell, a short period after the injection of labeled amino acid. Allfrey, Mirsky, and Osawa (*4*), using nuclei isolated from calf thymus and liver, observed that the protein most closely associated with DNA has the highest activity and the soluble ribonucleoprotein is also active, although histone is rather inert. In the present work, the rates of the incorporation of C^{14}-glycine into the protein moiety of desoxyribonucleoprotein fraction (DN-P), that of ribonucleoprotein fraction (RN-P), and the soluble protein fraction of normal and regenerating rat livers were investigated after the liver slices were incubated at 37° with C^{14}-glycine in Krebs-Ringer phosphate buffer. Interest was centered in the amino acid uptake by RN-P and DN-P of the regenerating liver slices for the reasons described above. Further, the effect of various pyrimidine and/or purine derivatives on the glycine uptake was studied.

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* A part of the present reports was presented at the Symposium on the Application of Radioisotopes on the Biochemistry held at the 27th Meeting of the Japanese Biochemical Society held in Kyoto on 2nd, April, 1955, and the remainder was presented at the Symposium on the Protein Biosynthesis at the 29th Meeting of the Japanese Biochemical Society held in Fukuoka on 30th, October, 1956.

EXPERIMENTAL

Treatment of Animals—Albino rabbits (2–3 kg.) or albino rats (100 to 120 g.) were used. The partial hepatectomy was performed by employing the method of Brues *et al.* (*5*), and one-half to two-thirds of the liver was removed. The partially hepatectomized animals were kept for 48 hours in the case of rabbits and for 20 hours in the case of rats. The rats received the nutritionally complete synthetic ration which contained 25 per cent of casein as a protein source, *ad libitum* for at least 4 days before the operation.

Incorporation Experiments—The animals were sacrificed by decapitation, the livers were perfused rapidly with cold 0.9 per cent saline, and the tissue slices were prepared by means of a Stadie-Riggs slicer in a cold room. In the experiment using a rabbit, 3 g. of liver slices were suspended in 4.6 ml. of slightly modified Krebs-Ringer phosphate solution (0.9 per cent NaCl, 100; 1.15 per cent KCl, 4; 3.84 per cent $MgSO_4 \cdot 7H_2O$, 1; 0.1 *M* sodium phosphate (pH 7.8), 6; 1.22 per cent $CaCl_2$, 3) in a Warburg flask and 0.4 ml. of 1-C^{14}-glycine in 0.9 per cent NaCl solution was added to the flask. The specific activity of 1-C^{14}-glycine used was 8.5 μc/mg. The additional compounds were added to each flask and the total volume of the solution in the flask was made to 5.0 ml. The flasks were then incubated with shaking in 100 per cent oxygen at 37°, usually for 2 hours. Immediately after incubation, the flasks were inserted into an ice-water to stop the reaction and 1 *M* NaCl was added to the flasks to adjust the final concentration to 0.4 *M* NaCl. The content of each flask was homogenized in a cold room, taken up in a centrifuge tube, and after freezing and thawing twice, the fractionation of tissue proteins was carried out, employing the slightly modified method of Griffin *et al.* (*6*) to obtain the ribo- and desoxyribonucleoprotein fractions and soluble protein fraction. Each protein fraction, after extraction with hot and cold trichloroacetic acid to remove nucleic acids was treated with alcohol and hot alcohol-ether (3 : 1). The purified protein fractions thus obtained were dried on metal dishes under infrared rays. The dried materials were weighed and their radioactivity was determined by the Lauritsen electroscope. Corrections were made for self absorption from an empirically determined curve. All these procedures were described in the previous paper (*7*).

In the experiments using a rat, 0.5 g. of liver slices was suspended in 2.2 ml. of the Krebs-Ringer phosphate solution described above and 0.1 ml. of 2-C^{14}-glycine in 0.9 per cent solution was added. The total volume of the solution in the flask was made to 2.5 ml. The specific activity of C^{14}-glycine used was 51.5 μc/mg. All other procedures were the same as described above. The incorporation data were expressed as microgram of C^{14}-incorporated into one gram of protein, which was calculated according to Farber, Kit, and Greenberg (*8*).

RESULTS

When the 1-C^{14}-glycine was incubated with regenerating rabbit liver slices under the conditions described above, the incorporation of C^{14}-glycine into each protein fraction proceeded for at least two hours as shown in Fig. 1. The rate of glycine uptake by these protein fractions of regenerating

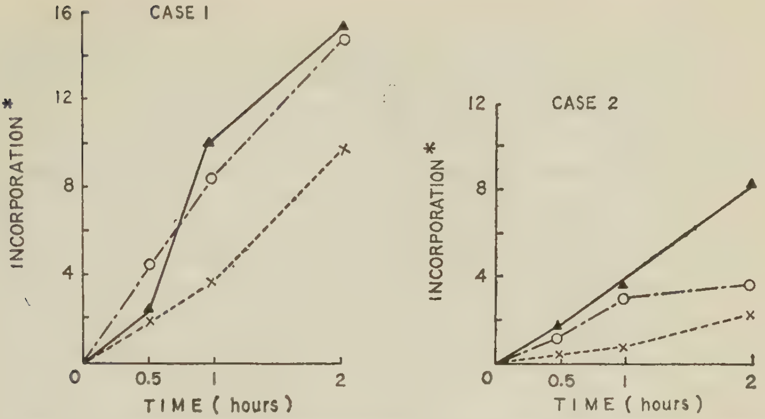

FIG. 1. Time curve of the incorporation of C^{14}-glycine into the protein fractions. —▲—, DN-protein fraction; —○—, RN-protein fraction; —×—, Soluble protein fraction. Incorporation* = div./min. per g. of protein/(M/12 × div./min. per μg. of glycine). The specific activity of C^{14}-glycine is 8.5 μc/mg. The incubation mixture contains 3 g. of rabbit liver slices in 5 ml. of incubation medium.

TABLE I

Incorporation of C^{14}-Glycine into the Protein Fractions of Normal and Regenerating Liver Slices

(1) Rabbit liver slices

Fraction	Incorporation*											
	Normal				Regenerating							
				(Mean)								(Mean)
Soluble protein	1.2	1.0	1.4	1.2	3.6	2.2	2.1	1.7	1.9	1.1	1.7	2.0
RN-protein	1.1	0.8	1.3	1.1	3.6	3.0	2.9	4.2	2.9	1.9	2.9	3.1
DN-protein	2.1	1.8	2.4	2.1	6.9	5.6	3.5	6.7	3.2	2.0	3.4	4.5

* Incorporation = div./min. per g. Protein/(M/12 × div./min. per μg. of glycine) The specific activity of C^{14}-glycine is 8.5 μc/mg. The incubation mixture contains 3 g. of liver slices in 5 ml. of incubation medium and the concentration of glycine is 2.25×10^{-3} *M*.

(2) Rat liver slice

Fraction	Incorporation*										
	Normal					Regenerating					
					(Mean)						(Mean)
Soluble protein	0.21	0.27	0.32	0.37	0.29	0.60	1.76	1.10	0.84	0.68	0.99
RN-protein	0.31	0.25	0.66	0.53	0.44	0.77	1.56	1.71	1.18	0.70	1.18
DN-protein	0.44	0·71	0.66	0.49	0.57	1.04	2.16	1.90	0.71	0.92	1.34

* Incorporation = div./min. per g. Protein/(M/12 × div. min. per μg. of glycine) The specific activity of C^{14}-glycine is 51.5 μc/mg. The incubation mixture contains 0.5 g. of liver slices in 2.5 ml. of incubation mixture and the concentration of glycine is 0.104×10^{-3} *M*.

liver slices was then compared with those of normal liver slices after a two hour incubation. As shown in Table I, the C^{14} incorporation into these protein fractions of both rabbit and rat livers slices than in the normal.

It was suspected that a portion of the radioactivities in C^{14}-glycine experiments might be attributed to glutathione bound to the protein by disulfide linkages as already pointed out by Peterson and Greenberg (*9*). To determine whether the above data on C^{14}-activities of the protein fractions were due mainly to peptide formation or considerable portions of the activities were due to C^{14}-glycine-labeled glutathione bound to the protein by disulfide linkages, the protein fractions were treated with performic acid to split the disulfide linkages by the modified method used by Peterson and Greenberg (*9*) of Sanger's (*10*) as follows: Each protein fraction was dissolved in 2 to 4 ml. of 98 per cent formic acid, 0.4 to 0.8 ml. of 30 per cent hydrogen peroxide was added, and after 30 minutes' standing at 37°, trichloroacetic acid was added to a final concentration of 5 per cent. The precipitated protein was washed as described in the preceeding paragraph. The percentage loss of the radioactivity of the protein fractions by this performic acid treatment (averages in three experiments) was zero in the soluble protein fraction, 3 per cent in the RN-P fraction, and 5 per cent in the DN-P fraction, showing that the radioactivity found in our experiments might be attributed mainly to the labeled carbon bound to the protein by a peptide bond.

Effect of Metabolic Inhibitors—The effect of various metabilic inhibitors (final concentration, 10^{-3} *M*) on the glycine uptake by the regenerating rabbit liver slices was examined (Table II). It was observed that azide and monoiodoacetate markedly inhibited the incorporation of C^{14}-glycine into each protein fraction. Arsenate in this concentration markedly inhibited the incorporation of C^{14}-glycine into RN-P fraction but its inhibitory effect on the incorporation of C^{14}-glycine into soluble protein and DN-P fractions was not observed. The effect of monoiodoacetate was the greatest and it inhibited almost completely the glycine incorporation into both soluble

TABLE II

Effect of Various Metabolic Inhibitors on the Incorporation of C^{14}-Glycine into the Protein Fractions of Regenerating Rabbit Liver Slices

Fraction	Inhibitors (final concentration, 10^{-3} *M*)			
	None	Na-azide	Na-arsenate	Monoiodoacetate
	Incorporation*			
Soluble protein	0.9	0.5	0.9	0
RN-protein	1.8	0.7	0.9	0.1
DN-protein	2.4	0.8	2.1	0

* Incorporation = div./min. per g. Protein/M/12 × div./min. per μg. of glycine
The experimental conditions are the same as in Table I (*1*).

protein and DN-P fractions in the final concentration of 10^{-3} *M*.

Effect of Purines and Pyrimidines—Newman and Grossman (*11*) reported that in partially hepatectomized rats, the rate of liver regeneration was significantly accelerated when fed on a diet added with ribo- or desoxyribonucleic acid, and some evidences (*12-15*) for uracil and thymine utilization in the formation of nucleic acids by regenerating liver or hepatoma were reported. Therefore, the effect of various purines and pyrimidines on glycine uptake by the protein fractions of regenerating liver slices was investigated. In the normal rabbit liver slices, thymine only slightly stimulated the glycine incorporation into RN-P and DN-P. On the contrary, in the regenerating liver slices, thymine stimulated significantly the glycine uptake by all these protein fractions (Table III). The effect of other pyrimidine derivatives on the glycine uptakes was then compared with that of

TABLE III

Effect of Thymine on the Incorporation of C^{14}-Glycine into the Protein Fractions of Normal and Regenerating Rabbit Liver Slices

Fraction	Normal liver slices		Regenerating liver slices	
	None	Thymine, 10^{-3} *M*	None	Thymine, 10^{-3} *M*
	Incorporation*			
Soluble protein	1.0	0.9	1.7	2.7
	1.4	1.5	1.9	2.0
			1.1	1.4
			2.2	2.9
			2.1	2.4
(Mean)	1.2	1.2 (+0%)	1.8	2.3 (+28%)
RN-protein	0.8	0.8	2.9	3.5
	1.3	1.8	2.6	2.9
			1.9	2.1
			3.0	3.8
			2.9	4.5
(Mean)	1.1	1.3 (+19%)	2.8	3.4 (+24%)
DN-protein	1.8	1.8	3.4	5.0
	2.4	3.4	3.2	5.4
			2.0	4.0
			5.6	8.7
			3.5	5.6
(Mean)	2.1	2.6 (+21%)	3.5	5.7 (+63%)

The figures in parentheses are per cent changes from control.

* Incorporation = div./min. per g. of protein/(M/12 × div./min. per μg. of glycine)

The experimental conditions are the same as in Table I (*1*).

TABLE IV

Effect of Pyrimidines on the Incorporation of C^{14}-Glycine into the Protein Fractions of Normal and Regenerating Rabbit Liver Slices

	Normal liver slices				Regenerating liver slices			
	None	Thymine	Uracil	Cytosine	None	Thymine	Uracil	Cytosine
Soluble protein	100	90	140	150	100	159	170	200
	100	107	107	121	100	105	110	152
					100	127	110	127
	Mean	99	124	136	Mean	130	133	160
RN-protein	100	100	100	112	100	121	121	121
	100	138	108	138	100	111	111	138
					100	126	158	163
	Mean	119	104	125	Mean	119	138	141
DN-protein	100	100	100	223	100	147	109	129
	100	142	158	225	100	169	122	134
					100	200	155	185
	Mean	121	129	224	Mean	172	129	149

The final concentration of each addition compound is 10^{-3} *M*. All data are the incorporations expressed as percentages to control. The experimental conditions are the same as in Table I (*1*).

TABLE V

Effect of Pyrimidines on the Incorporation of C^{14}-Glycine into the Protein Fractions of Regenerating Rat Liver Slices

Fraction	Pyrimidines (final concentration, 10^{-3} *M*)			
	None	Thymine	Thymine + Uracil	Thymine + Uracil + Cytosine
Soluble protein	100	123	108	83
	100	127	109	130
	100	129	145	148
	Mean	126	121	121
RN-protein	100	135	120	85
	100	134	119	151
	100	129	154	204
	Mean	133	131	147
DN-protein	100	134	104	79
	100	144	105	181
	100	106	120	179
	Mean	128	110	146

All date are the incorporations expressed as percentages to control. The experimental conditions are the same as in Table I (*2*).

TABLE VI

Comparison of the Effect of Pyrimidine and Purine Mixture on the Incorporation of C^{14}-Glycine into the Protein Fractions of Regenerating Rat Liver Slices

Fraction	Addition compound (final concentration, 10^{-3} M)			
	None	Pyrimidine mixture*	Purine mixture**	Pyrimidine and purine mixture
Soluble protein	100	117	102	108
	100	125	100	111
	100	105	101	104
	Mean	116	101	108
RN-protein	100	129	114	127
	100	133	121	135
	100	127	113	127
	Mean	130	116	130
DN-protein	100	138	141	161
	100	125	132	148
	100	130	130	149
	Mean	131	134	153

All data are the incorporations expressed as percentages to control.
* Thymine, uracil and cytosine
** Adenine and guanine
The experimental conditions are the same as in Table I (*2*).

TABLE VII

Comparison of the Effect of Thymine and Orotic Acid on the Incorporation of C^{14}-Glycine into the Protein Fractions of Regenerating Rat Liver Slices

Fraction	Addition compound (final concentration, 10^{-3} M)		
	None	Thymine	Orotic acid
Soluble protein	100	194	221
	100	203	229
	100	200	218
	Mean	199	223
RN-protein	100	200	216
	100	229	221
	100	214	241
	Mean	214	233
DN-protein	100	188	182
	100	223	220
	100	198	180
	Mean	203	194

All data are the incorporations expressed as percentages to control. The experimental conditions are the same as in Table I (*2*).

thymine. As shown in Table IV, the addition of uracil or cytosine in the regenerating rabbit liver slices was effective in accelerating the uptake, and the effect was also observed in the normal liver slices. Next, the effect of thymine alone and a pyrimidine mixture (thymine and uracil, or thymine, uracil, and cytosine) on the glycine uptake by each other. They all enhanced the glycine uptake, but significant additive effect of these compounds was not observed (Table V). Then the effect of a pyrimidine mixture (thymine, uracil and cytosine) was compared with that of purine mixture (adenine and guanine) on the incorporation of glycine into the regenerating rat liver slices. As represented in Table VI, the pyrimidine mixture especially stimulated the glycine incorporation into DN-P and RN-P, while the purine mixture did that into DN-P. The simultaneous adddition of the pyrimidine and purine mixtures showed the additive effect of the increase of the glycine incorporation into DN-P. The addition of orotic acid, which is known as a precursor of the nucleic acid pyriminine, was furhter studied using the regenerating rat liver slices and its stimulating effect was compared with that of thymine (Table VII). As shown in Table VII, orotic acid greatly stimulated the glycine uptake by each protein fraction and it was remarkable that the stimulating effect of thymine and orotic acid on the glycine incorporation was almost the same in these cases.

DISCUSSION

The conclusive evidence has not yet been presented which answers whether the amino acid incorporation into protein *in vitro* occurs by *de nove* synthesis of protein from acids, or only by a partial exchange of amino acids in the protein molecule with added C^{14}-amino acids in the medium, but it may be postulated that the incorporation rate of amino acids means the metabolic activity of the protein, especially when the same protein fraction is compared under the different experimental conditions. In the present study the enhanced uptake of amino acid by protein moieties of nucleoproteins of the regenerating liver slice was observed as compared with that of the normal liver slices. There have been many reports (*17–22*) which show that the renewal rate of nucleic acid was enhanced in regenerating liver as compared with that of resting liver. Our results indicate that the metabolic activity of the protein moieties of nucleoprotein is related in some degree to that of nucleic acid in the regenerating liver.

It should be further noted that the C^{14}-glycine uptake by DN-P was of the same order as that by RN-P or soluble protein even in the case of normal rat or rabbit liver slices, although it had already been shown by many workers (*18*, *23–28*) that desoxyribonucleic acid (DNA) was rather metabolically inert especially in the nonproliferating animal liver. The above results may suggest that the protein moiety of DN-P is metabolically active, although it remains indefinite until further purification of DN-P is carried out. In this respect, the recent work of Allfrey *et al.* (*4*) attracts the authors' attention. In this work, the C^{14}-alanine uptake by various protein fractions was estimated and the highest incorporation was observed in non-histone protein associated with DNA, although that of histone showed a very low value.

The metabolic inhibitors which inhibit the tissue oxidation and the oxidative phosphorylation were shown to inactivate the amino acid incorporation into each protein fraction of the regenerating rabbit liver, as shown by many workers (*29*) employing the whole of the liver slices. This fact may mean that the incorporation of amino acids into the peptide bonds of proteins must require the formation of an energy-rich phosphate bond. The inactivating effect of monoiodoacetate was the most powerful among the inhibitors examined. There have been reports (*30*, *31*) which suggest that SH radicals take part in the process of cellular mitosis. The powerful inhibition of monoiodoacetate may be interpreted from this aspect.

It should be noted that pyrimidines such as thymine, uracil, or cytosine accelerated the amino acid incorporation into each protein fraction of the regenerating liver slices. The precise mechanism of these accelerations must be clarified by further studies, but some suggestive indications may be given from the standpoint of the participation of nucleic acid in the protein synthesis. It is now widely accepted that ribonucleic acid (RNA) and DNA play an essential role in a protein synthesis. Therefore, to discuss our experimental results, it becomes very important whether pyrimidines and purines are utilized or not for the nucleic acid synthesis in the regenerating liver. As to the pyrimidines, there have been some literatures (*12–14*) indicating that C^{14}-uracil as well as C^{14}-thymine are incorporated into nucleic acid in proliferating animal tissues, although it was shown by Plentl and Schoenheimer (*32*), and Bendich *et al.* (*33*), that the injection of N^{15}-labeled pyrimidines into normal adult rats does not lead to appreciable labeling of the total nucleic acid. Rutman *et al.* (*13*) investigated that uracil-2-C^{14} was incorporated into the nucleic acids of rat hepatoma induced by 2-acetylamino-fluorene to a considerably greater extent than into those of the normal liver. Furthermore, Heidelberger *et al.* (*14*) have reported recently that in the liver of a normal rat some incorporation of uracil into the acid-soluble-and nucleic acid-pyrimidines occur to only a small but noticeable extent. In the livers of tumor bearing rats, however, there is a several-fold increase in its utilization, and degree of its utilization is comparable with that of C^{14}-orotic acid*.

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* After this report had been written, Canellakis has demonstrated that the soluble cytoplasmic fraction of rat liver contains uridine phosphorylase which catalyzes the reversible reaction: uracil + ribose phosphate = uridine + phosphate, and uridine kinase which phosphorylates uridine to form uridine-5′-phosphate, in the presence of adenosine triphosphate and Mg^{++}. The existence of enzymatic pathways in the liver which can convert uracil to the nucleotide stage contrasts with the limited ability of this organ to incorporate uracil into RNA. It has also been shown that the ability to incorporate C^{14}-2-uracil into RNA of regenerating rat liver slices is greater than that of normal rat liver slices, while decreased capacity to degrade C^{14}-uracil to $C^{14}O_2$ is demonstrated in regenerating rat liver slices compared to normal rat liver slices. (Canellakis, E. S., *J. Biol. Chem.*, **227**, 329, 701, 1957)

As to thymine, it was shown by Holmes *et al.* (*15*) that thymine-1-C^{14} was utilized *in vivo* to a small but significant extent for the synthesis of DNA by regenerating rat liver. Recently we have also been able to demonstrate that thymine-2-C^{14} is incorporated to some extent into DNA of regenerating rat liver slices (*16*). In this respect, it is interesting that thymine significantly increased C^{14}-glycine uptake into the protein fractions in the regenerating rabbit liver slices and had no significant effect on the normal rabbit liver slices. Orotic acid is now generally accepted as a main precursor of the RNA pyrimidines in animal tissues (*34*). Therefore, its effect on the incorporation of C^{14}-glycine may be well explained from the increased requirement of orotic acid as a pyrimidine precursor for increased RNA synthesis.

There have been several reports (*34–39*) concerning the utilization of adenine as the precursor of nucleic acids in animal tissues. It was especially observed by Balis *et al.* (*22*) that there was a greatly increased uptake of adenine-C^{14} into RNA of regenerating liver as compared with that of control animals.

Therefore the observed effect of the pyrimidines and purines may be interpreted as the result of the enhanced requirement of pyrimidines and purines as the precursors of nucleic acids in the regenerating liver slices. These experimental results may demonstrate more directly the *in vivo* observation of Newman and Grossman (*11*) in which the rate of liver regeneration in partially hepatectomized rats was significantly accelerated when fed on nucleic acid rich diet.

SUMMARY

The incorporation of 1-C^{14}-glycine or 2-C^{14}-glycine into the protein moieties of ribo- and desoxyribonucleoprotein fractions as well as the soluble protein of the normal and regenerating livers of rabbits or rats was studied after the incubation of liver slices at 27° with labeled glycine in Krebs-Ringer medium. The following results were obtained.

1. The percentage loss of radioactivity of the protein fractions by the performic acid treatment was zero in the soluble protein fraction, 3 per cent in the RN-P fraction, and 5 per cent in the DN-P fraction, showing that the radioactivity found in our exeriments might be attributed mainly to the labeled carbon bound to the protein by peptide bond.

2. The increased incorporation of glycine into each protein fraction of the regenerating liver slices was observed as compared with that of normal slices.

3. Arsenate, 2,4-dinitrophenol, and monoiodoacetate (each in 10^{-3} M in final concentration) markedly inhibited the glycine utake of each protein fraction of the regenerating rabbit liver slices and the inhibitory effect of monoidoacetate was the greatest.

4. The addition of thymine in the reaction medium stimulated the glycine uptake by regenerating liver protein of rabbit and rat. The stimulat-

ing effect of thymine was of the same degree as that of orotic acid.

5. The addition of uracil or cytosine increased the incorporation of glycine into each protein fraction of regenerating liver slices, its effect on the protein fractions of the normal liver slices being less.

6. The addition of pyrimidine mixture accelerated the glycine uptake by the regenerating liver slices, but no additive effect of each pyrimidine compound was observed, while the addition of purine mixture stimulated particularly the incorporation of glycine into the protein moiety of desoxyribonucleoprotein of the regenerating liver slices. Simultaneous addition of both purine- and pyrimidine mixtures accelerated the incorporation of glycine into the protein moieties of ribo- as well as desoxyribonucleoproteins of the regenerating liver slices.

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STREPTOLYSIN-FORMING ACTIVITY OF ENZYMATICALLY SYNTHESIZED POLYNUCLEOTIDES AND OF RIBONUCLEIC ACIDS PREPARED FROM AZOTOBACTER VINELANDII AND TOBACCO MOSAIC VIRUS[1]

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The nature of the active substance from yeast RNA[3] effective in the streptolysin formation by *Streptococcus hemolyticus* has been studied in our laboratory (*1–7*). As already reported, it has been shown that the remarkable enhancement of streptolysin-forming activity of yeast RNA caused by the action of pancreatic RNase is due to the specificity of the enzyme, especially the formation of the RNase-limit oligonucleotides which are rich in guanylic acid.

Besides these studies on yeast RNA, it was desirable to test the potentialities of other RNAs. The polynucleotides synthesized from nucleoside-5′-diphosphates by polynucleotide phosphorylase seemed to be very suitable for this purpose, since by this method it is possible to prepare polynucleotides possessing widely different base compositions especially those containing only one base or any combination of bases (*8–11*). RNA prepared from *Azotobacter vinelandii* was also of some interest as it is derived from the bacterium from which the polynucleotide phosphorylase was obtained. RNA from TMV was also included in our study in view of the inactivity of its RNase core as reported by Bernheimer (*12*).

The present paper deals with the studies on the streptolysin-forming activity of these RNAs and polynucleotides. Some of the results described in this paper has briefly been reported in collaboration with Ochoa, Bernheimer and others (*4*).

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[3] The following abbreviations are used: ADP, adenosine diposphate; CDP, cytidine diphosphate; DNA, desoxyribonucleic acid; GDP, guanosine diphosphate; RNA, ribonucleic acid; RNase, pancreatic ribonuclease; TMV, tobacco mosaic virus; Tris, tris (hydroxymethyl)-aminomethane; UDP, uridine diphosphate.

EXPERIMENTAL

Materials

The following polynucleotide and RNA preparations were employed in the present investigation. Among these, all of the enzymatically synthesized polynucleotides and RNA prepared from *Azotobacter vinelandii* were kindly supplied from Professor S. Ochoa of New York University.

Poly AGUC (1 : 1 : 1 : 1): Enzymatically synthesized from an equimolar mixture of ADP, GDP, UDP and CDP (*10*).

Poly AGUC (1 : 0.5 : 1 : 1): Enzymatically synthesized from a mixture of ADP, GDP, UDP, and CDP; the molar ratio of these nucleoside diphosphates being 1 : 0.5 : 1 : 1 (*10*).

Poly AU: Enzymatically synthesized from an equimolar mixture of ADP ann UDP (*10*).

Poly AC: Enzymatically prepared from an equimolar mixture of ADP and CDP (*10*).

Poly A: Enzymatically prepared from ADP (*10*).

Poly GUC: Enzymatically prepared from GDP, UDP and CDP; the molar ratio of these nucleoside diphosphates being 0.5 : 1 : 1 (*10*).

RNA from Azotobacter vinelandii: (*10*).

Yeast RNA: Prepared by Clarke and Schryver's method. The sodium salt was exhaustively dialyzed against distilled water and then lyophilized.

RNase Core of Yeast RNA: Prepared from Schwarz's yeast RNA as previously reported (*7*).

RNA frome TMV: Prepared as previously reported (*13*).

RNase Core of TMV-RNA: Prepared as previously reported (*13*).

The base analysis of these preparations are summarized in Table I.

TABLE I
Base Composition of Polynucleotides

Preparations	Base composition			
	Adenine	Guanine	Uracil	Cytosine
Poly AGUC (1 : 1 : 1 : 1)*	10	11.3	6.4	5.9
Poly AGUC (1 : 0.5 : 1 : 1)*	10	6.2	6.8	6.8
Poly GUC (0.5 : 1 : 1)*		10	10.5	11.4
RNA from *Azotobacter vinelandii***	10	13	7.3	9
Yeast RNA***	10	11.2	9.8	7.9
RNase core of yeast RNA***	10	17.8	4.2	4.4
RNA from TMV***	10	8.7	10.1	6.7
RNase core of TMV-RNA***	10	5.6	2.8	4.5

* These figures were taken from the data obtained in Prof. Ochoa's laboratory,
** Analytical data were taken from Mii (*14*).
*** Obtained in our laboratory by the method previously reported (*7*).

Methods

Ribonuclease Digestion—Samples were dissolved in *M*/20 Tris buffer, pH 7.5. The solution containing 1.0 mg. of sample was digested at 37° for 2 and 4 hours with 0.05 mg. of crystalline pancreatic RNase in a final volume of 1.0 ml. Control experiments were similarly run for 4 hours without RNase. After the incubation, the samples were lyophilized and subjected to the determination of potency. Only in the case of TMV-RNA, the digestion was carried out for 2 hours with 0.05 mg. and 0.005 mg. of RNase in view of the inactivity of RNase core of TMV-RNA. Accordingly the control experiment was run for 2 hours without enzyme.

Determination of Potency—The essentially same method as described in the previous paper (*2*) was employed in the present investigation. The assay was always conducted with the RNase digest as well as the control sample. RNase core of yeast RNA and TMV-RNA were, however, directly assayed without RNase digestion. It was empirically known that the time of incubation with resting *Streptococcus hemolyticus* cells required for the maximum hemolysin formation varies for different polynucleotide preparations. Five or four measurements were therefore carried out in the course of 2 hours incubation at given intervals.

Paper Chromatography—Paper chromatography was carried out with *iso*-propanol-water-ammonia as solvent (*15*).

RESULTS AND DISCUSSION

TABLE II

Effect of Polynucleotides and Their Ribonuclease Digestion Products on Streptolysin Formation by Resting Cells of Streptococcus Hemolyticus

Samples	Concentration in assay (*μg./ml.*)	Time of ribonuclease digestion (*hrs.*)	Activity (*H.U.*)
Poly AGUC (1 : 1 : 1 : 1)	400	0*	226
	40	2	447
	40	4	491
Poly AGUC (1 : 0.5 : 1 : 1)	400	0*	0
	40	2	33
	40	4	39
Poly GUC (0.5 : 1 : 1)	400	0*	0
	40	4	119
Poly AU (1 : 1)	400	0*	0
	100	4	0
Poly AC (1 : 1)	400	0*	0
	100	4	0
Poly A	400	0*	0
	100	4	0
GMP	400	—**	0
GDP	800	—**	0
	200	4	0
Yeast RNA	400	0*	10
	40	2	140
	40	4	171
RNase core of yeast RNA	20	—**	1140
RNA from *Azotobacter vinelandii*	400	0*	10
	20	4	172
RNA from TMV	400	0***	0
	100	2	72
	100	2****	48
RNAase core of TMV-RNA	100	—**	0
	40	—**	0

* Incubated at 37° for 4 hours without RNase.
** Activity was measred directly.
*** Incubated at 37° for 2 hours without RNase.
**** Only in this case, digestion was carried out with 1/10 quantity of the RNase used in other experiments.

As shown in Table II, poly AGUC(1 : 1 : 1 : 1) was highly active for streptolysin formation, while poly AGUC (1 : 0.5 : 1 : 1) which contains less guanylic acid residues possessed only neglegible activity (*cf.* Fig. 1). Similarly,

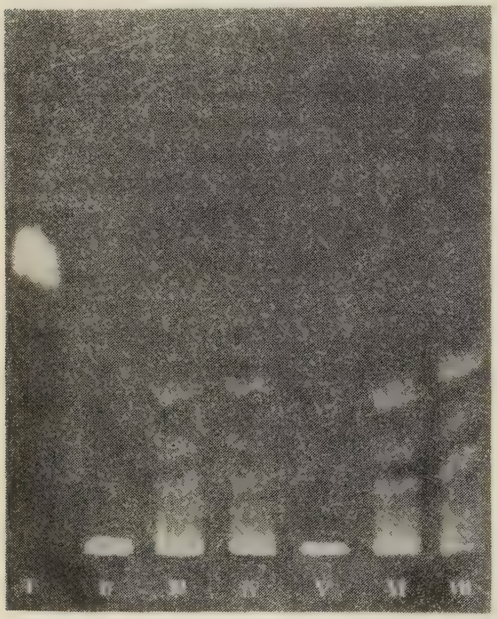

Fig. 1. Ribonuclease digestion of AGUC (1 : 1 : 1 : 1) and AGUC (1 : 0.5 : 1 : 1)

The solution containing 1.0 mg. of sample was digested at 37° with 0.05 mg. of crystallin RNase.

I: Yeast adenylic acid.

II: AGUC (1 : 1 : 1 : 1) incubated at 37° for 4 hours without RNase.

III: AGUC (1 : 1 : 1 : 1) digested for 2 hours.

IV: AGUC (1 : 1 : 1 : 1) digested for 4 hours.

V: AGUC (1 : 0.5 : 1 : 1) incubated at 37° for 4 hours without RNase.

VI: AGUC (1 : 0.5 : 1 : 1) digested for 2 hours.

VII: AGUC (1 : 0.5 : 1 : 1) digested for 4 hours.

Solvent system: *iso*-Propanol-H_2C-NH_3 (*15*).

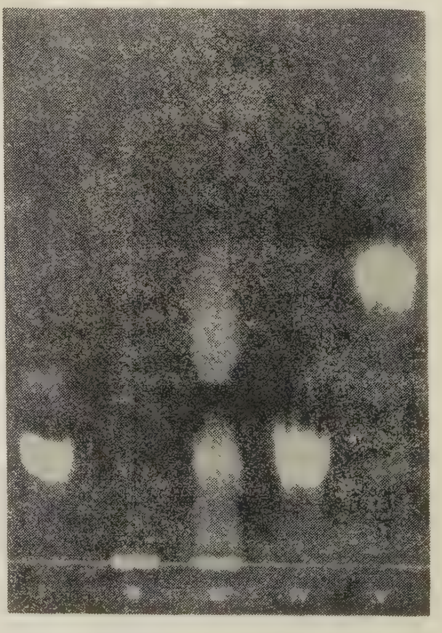

FIG. 2. Ribonuclease digestion of GUC (0.5 : 1 : 1).

Conditions were the same as in the experiments of Fig. 1.

I: GMP (guanosine-5′-monophosphate)

II: Poly GUC incubated at 37° for 4 hours without RNase.

III: Poly GUC digested for 4 hours.

IV: GDP (guanosine-5′-diphosphate).

V: Yeast adenylic acid.

Solvent system: *iso*-Propanol-H_2O-NH_3 (*15*).

although yeast RNA was quite active, TMV-RNA which is poor in guanylic acid showed little or no activity. RNA from *Azotobacter vinelandii*, the base composition of which resembles that of AGUC (1 : 1 : 1 : 1), was also highly active. The polynucleotides containing no guanylic acid residue were all inactive. The poly GUC was also effective for hemolysin formation as expected, while GDP and GMP were completely inactive (*cf.* Fig. 2).

These facts strongly supported the conclusion obtained from the fractionation studies of active substance from yeast RNase core (*7*) and confirmed the importance of oligonucleotides relatively rich in guanylic acid residue for streptolysin formation.

SUMMARY

1. Streptolysin-forming activity of polynucleotides enzymatically synthesized from nucleoside diphosphates as well as RNAs obtained from *Azotobacter vinelandii* and TMV were studied.

2. High activity was observed with those polynucleotides which were rich in guanylic acid. The polynucleotides containing no guanylic acid residue were all inactive.

3. *Azotobacter vinelandii* RNA containing relatively large amounts of guanylic acid residue was highly active after RNase digestion, while the TMV-RNA containing smaller amounts of that residue showed only a little activity.

The authors wish to express their sincere thanks to Prof. S. Ochoa of New York University for kindly supplying a number of valuable samples and for informing the authors of the data of their base analyses.

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SPECTROPHOTOMETRY OF INTACT BIOLOGICAL MATERIALS

ABSOLUTE AND RELATIVE MEASUREMENTS OF THEIR TRANSMISSION, REFLECTION AND ABSORPTION SPECTRA

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There has been more or less uncertainty as to the observed amount of light absorbed by translucent biological materials. The difficulties of the absolute measurement come from the optical characteristics of translucent materials, which contrast with the properties of transparent materials in the following respects.

Firstly, translucent materials reflect light, while transparent materials do not. The principle of absorption spectrophotometry of transparent materials is based on the fact that unabsorbed light comes out from them only in the form of transmitted light, so that the measurements of incident and transmitted light give us the amount of absorbed light. This principle does not apply to translucent materials, in which we have to measure the amount of reflected light, in addition to the above quantittes, for calculating the amount of the absorbed light.

Secondly, incident parallel light is scattered by translucent materials. The diffuse nature of the transmitted and reflected light has made not only the absolute measurements but also the relative measurements more difficult than the measurement of the parallel light transmitted through transparent materials.

The purpose of this report is to present new techniques for measuring absolute and relative values of the transmittance and reflectance of biological samples, with a general theory on the spectrophotometry of translucent and non-transparent materials. Recently, Shibata, Benson and Calvin (*1*) developed a new method for observing the transmittance of translucent materials, using pieces of opal glass, and the method has been successfully applied for the measurement of biological samples (*1–6*). In the reports cited, however, the absoluteness of the value of the transmittance obtained by the method was not completely checked and the applications of the technique and its use as a relative measurement were mainly described. In the present study, the technique was further improved for obtaining absolute values and a complete theory on the technique was developed. The absoluteness of the transmittance of a sample observed by the improved method was checked in relation to the reflectance of the same sample. In the following description, the method will be called the *opal glass transmission method.* For observing absolute values of the diffuse reflectance of translucent samples of biological materials, another new method of using opal glass was recently presented by Shibata (*7*). This method will be referred to as the *opal glass reflectiou method.*

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These newly developed methods have the following advantages. i) They are independent methods, which give us directly the absolute values of transmittance and reflectance. From the transmission and reflection spectra of a biological sample observed by these methods, the spectrum on the basis of true absorption can be accurately calculated. ii) They are simple and practicable, because the small pieces of opal glass can be used with most of the commonly available spectrophotometers.

For observing the relative value of the reflectance of biological samples simply and conveniently, the opal glass reflection method was modified. As will be seen later, the reflection spectra of various translucent or non-transparent materials observed by this modified method showed clear absorption bands. As one of the application of this technique, the state of carotenoids *in vivo* in roots or fruits was studied.

Instead of using the units of transmittance and reflectance, the logarithms of their reciprocals were mainly used. They are designated as *attenuance* and expressed by E throughout this study. The new word, attenuance, was adopted for translucent materials for expressing the quantity due to both absorption and scattering. It corresponds to absorbance (optical density) for transparent materials, where the quantity depends on only the absorption.

I. Principle of the Opal Glass Transmission Method

When the spectrum of a translucent biological material is measured with a commonly used spectrophotometer by the transmission method, usually the spectrum obtained has a rather high level in attenuance units, upon which are superimposed more or less indistinct diffuse absorption bands, whereas the spectrum of a solution of the pigments extracted from the sample has corresponding sharp absorption bands. A typical example is shown in Fig. 1 (*1*), where curve A is the spectrum of a suspension of the unicellular alga, *Chlorella*, and Curve C is the spectrum of the ethanol extracts from the suspension, both observed with a Cary Recording Spectrophotometer, a commonly used type.

By the opal glass transmission method, the spectrum of the suspension is greatly improved (Curve B in Fig. 1); namely i) the definition of the spectrum is remarkably increased, ii) the level (base line) where there is no absorption band is greatly reduced to a low horizonntal line, although the level is higher than the zero line of the spectrum, and iii) the attenuance measured is independent of the optical arragement used.

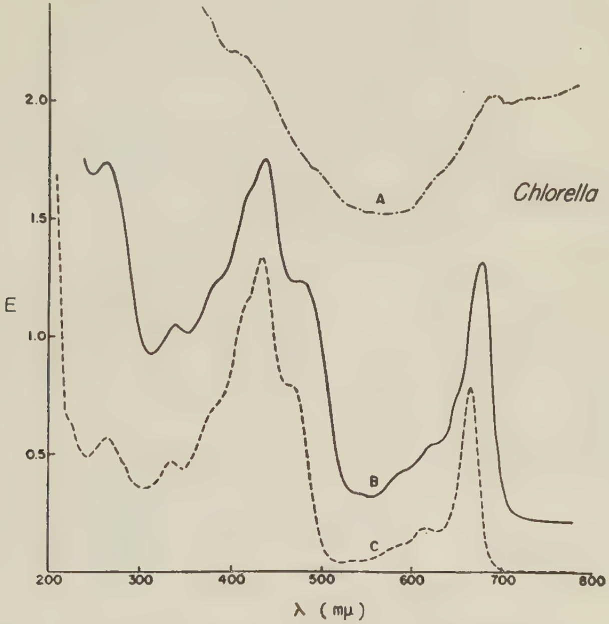

FIG. 1. Absorption spectra of chlorella suspension and extract; reproduced from the figure in ref. 1.

Curve A, cell suspension, without opal plate; Curve B, the same suspension with opal plate; Curve C, alcohol extract from the suspension.

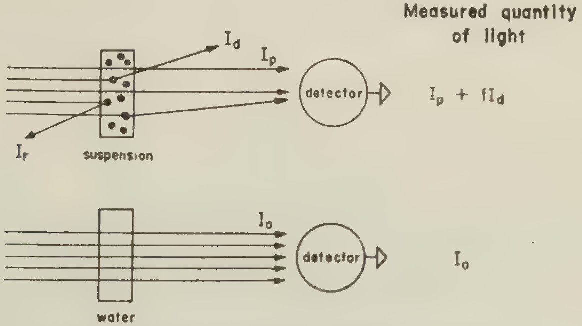

FIG. 2. Path of light in ordinary technique.

The principle of the opal glass transmission method can be explained easily, when it is known why poor results are obtained by ordinary techniques. Fig. 2 shows the path of light when the suspension is illuminated by parallel light. Roughly divided, two kinds of light are transmitted through the suspension. One is parallel light, which has passed through only the suspending medium without hitting the microorganisms. This light will be called *parallel transmitted light* and its amount will be expressed by I_p. The other kind is diffused light, which has hit the cells of microorganisms. This light will be called *diffuse transmitted light* and its amount will be denoted by I_d. Therefore, the amount of total transmitted light (I_t) is

$$I_t = I_p + I_d \qquad (1)$$

in addintion to these light beams, we have reflected light coming out of the sample, which also has hit the cells of microorganisms. Obviously, the following relation exists between these beams;

$$I_0 = I_a + I_r + I_t \qquad (2)$$

where I_0, I_a and I_r stand for the amounts of incident, absorbed and reflected light, respectively.

In ordinary spectrophotometers there is always a certain distance between the sample and the light detector (See Fig. 2). The intensity of the diffuse transmitted light decreases until it reaches the detector, while the parallel transmitted light does not decrease in intensity. Therefore, in the measurement by ordinary spectrophotometers, the light emerging in a direction not intercepted by the photocell is totally left out of account. What we are measuring in E units is the following quantity, which may be designated as *quasi-attenuance* (${}_pE_{pfd}$);

$${}_pE_{pfd} = \log \frac{I_0}{I_p + fI_d}\,;\ 0 < f < 1 \qquad (3)$$

where f is a constant between 0 and 1. The subscript p to the left of E indicates the nature of the reference incident light (parallel light), and subscripts p, f and d on the right side express the nature of the light transmitted by the sample and actually captured by the detector (f and subscripts p and d in the denominator in the above equation).

When f is equal to 0 or 1, equation (3) becomes equations (4) or (5), respectively;

$${}_pE_p = \text{long}\, \frac{I_0}{I_p} \qquad (4)$$

$${}_pE_{pd} = \text{long}\, \frac{I_0}{I_p + I_d} \qquad (5)$$

we shall call ${}_pE_p$ and ${}_pE_{pd}$, *recti-linear attenuance* and *semi-integral attenuance* with parallel illumination, respectively. ${}_pE_p$ is the attenuance on the basis of parallel transmitted light and ${}_pE_{pd}$ is the attenuance on the basis of total transmitted light.

The value of f in equation (3) depens upon the geometry of the optical system, especially upon the distance between the sample and the light detector, and also depends on the diffuseness of the diffuse transmitted light. Under the condition employed in most commonly used spectrophotometers, the value of f is quite small, a situation that makes the general level (base line) of measured spectra considerably higher than it would be in the spectra in semi-integral attenuance units.

The lack of definition in the spectra by ordinary techniques is also due to the smallness of the factor, f, in equation (3). As the diffuse transmitted light is the light, which has hit the cells but was not absorbed, I_0/I_d varies with the wavelength depending upon the light absorption by the colored substances in suspended particles. On the other hand, I_0/I_p is independent of wavelength, since parallel transmitted light does not have any characteristics imparted to it by the pigments in suspended particles. Therefore, quasi-attenuance with a small value of f may not vary so markedly with wavelength as does semi-integral attenuance. As is clear from equation (3), the best value of f for obtaining a low base line and sharp absorption bands is 1.

The opal glass transmission method cited above (*1*) is a simple technique for measuring *approximate* values of semi-integral attenuance of translucent materials with parallel-light illumination. The principle is illustrated in Fig. 3. A plate of opal glass is attached to the detector side of the sample and reference cells, so that both the parallel and the diffuse light beams transmitted through a sample hit the opal plate and are completely diffused by the plate. Therefore, the same portion, α of the parallel and diffuse transmitted lights hitting the plate goes into the detector after being diffused by the opal plate. As will be explained later, some of the multiply reflected light between the sample and the opal plate also goes into the detector in addition to the purely transmitted light. If we neglect the effect of these multiply reflected beams, the amount of light measured on the sample side is $\alpha\,(I_p+I_d)$. By the same principle, the measured quantity on the reference side is αI_0, provided that optical characteristics of the opal plate and the optical system on the reference side are the same as those on the sample side. Therefore, the observed attenuance by the opal glass transmission method is semi-integral attenuance, when the effect of multiple reflection is small.

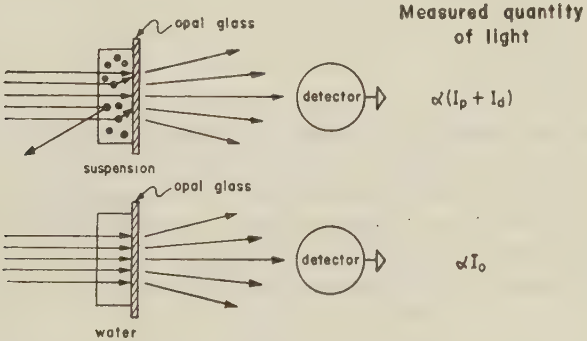

FIG. 3. Path of light in opal glass transmission method for the observation of ${}_pE'_{pd}$.

To the same degree of approximation, semi-integral attenuance with diffuse-light illumination can be measured also by using opal glasses (Fig. 4).

An opal plate is attached to the monochromator side of both the sample and reference cells, so that the sample is illuminated by the light diffused by the opal plate. Since the light emerging from the sample and the reference cells has been completely diffused, the value of E thus measured is semi-integral attenuance with diffuse-light illumination, ${}_dE_{pd}$, neglecting the effect of multiple reflection*.

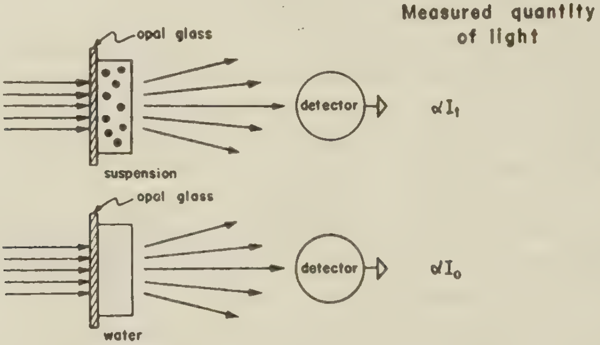

FIG. 4. Path of light in opal glass transmission method for the observation of ${}_dE_{pd}'$.

So far the principle of the opal glass transmission method has been illustrated by the example of a dilute suspension. When the concentration of a suspension is high, there is no parallel transmitted light, so that the effect of using opal glass appears only as a reduction of the base-line height, with no increased definition of the spectrum. One example is the observation of cytochrome bands of yeast between 500–600 mμ. For this we need a highly concentrated suspension to detect the weak bands of the cytochromes, which are present in small quantities in yeast cells. However, in the observations of suspensions of most microorganisms, the optimum concentration is rather low, so that we have both parallel and diffuse transmitted light.

The transmitted light from biological materials other than suspensions of cells is more complicated. Such examples are seen in leaves, petals and slices of tissues. The light transmitted through the the rather transparent part of such samples is similar to parallel light transmitted by suspensions, and the transmitted light which has hit rather dense particles, like nuclei or chloroplasts, is highly diffused. Actually, the transmitted light is composed of many kinds of beams with different degrees of diffuseness. The use of opal glass is quite effective for such samples (*1*, *2*, *6*). The effectiveness can be explained in a similar way as for a dilute suspension, by increasing the number of terms in the denominator of equation (3).

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* The transmitted light with diffuse-light illumination is completely diffused, having no parallel rays. However, it is composed of two kinds of light, that which has and has not hit the cells of suspended particles, as is the case with parallel-light illumination. From this point of view, subscript p was retained in the expression of ${}_dE_{pd}$.

II. Correction for Multiple Reflection

For correcting the effect of multiple reflection, let us first estimate the amount of the effect in the measurement of ${}_dE_{pd}$ with diffuse-light illumination. The amount of each multiply reflected beam is shown schematically in Fig. 5. In the actual system, the direction of the incident parallel beam is normal to the plane of the opal glass and only the incident light is parallel, although each light path is indicated by a single slanted arrow in the figure. Symbols R and T mean reflectance and transmittance for diffuse-light illumination, R' and T' for parallel-light illumination, and subscripts 1 and s refer to one sheet of opal glass and the sample, respectively. In Fig. 5, it is assumed that the light, once it goes through a sheet of opal glass, is completely diffused.

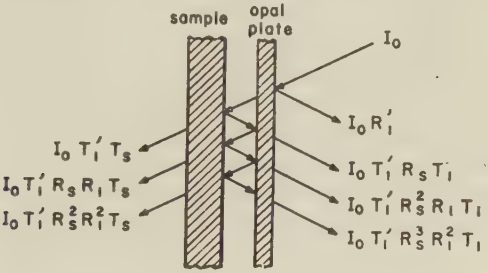

FIG. 5. Intensities of multiply reflected light between an opal plate and a sample.

Summing up the amounts of all beams on the left hand side of Fig. 5, the following equation is obtained for the transmittance (T_{1+s}) of the system as a whole.

$$T_{1+s} = \frac{T_1' T_s}{1 - R_1 R_s} \tag{6}$$

The transmittance of an opal plate on the reference side is obviously T_1'. Therefore, the value of attenuance measured by the opal glass transmission method with no correction for multiple reflection is

$${}_dE'_{pd} = \log \frac{1 - R_1 R_s}{T_s} = {}_dE_{pd} + \log(1 - R_1 R_s) \tag{7}$$

where a prime on E indicates that the effect of multiple reflection is not corrected. The term $\log(1 - R_1 R_s)$ in the above equation is due to the effect mentioned above. As will be shown later, the value of R_1 is approximately 0.5. Therefore, the difference between ${}_dE'_{pd}$ and ${}_dE_{pd}$ is 0.3, when R_s is 1.0. If R_s is 0.1, the difference is only 0.02, so that the correction is significant only when the sample is highly light-reflecting.

The correction for multiple reflection was carried out experimentally. A gray film in addition to an opal plate was placed on both sample and reference sides. On the sample side, the gray film was sandwiched between the sample and the opal plate. If we can assume that the gray film absorbs but does not reflect light, the observed attenuance (${}_dE''_{pd}$) with this system will be

$$ {}_dE''_{pd}=\log\frac{1-R_1R_sT_f^2}{T}={}_dE_{pd}+\log(1-R_1R_sT_f^2) \qquad (8) $$

where T_f is the transmittance of the gray film with diffuse-light illumination. If T_f is small enough, the second term in the above equation will be practically zero. Therefore, ${}_dE''_{pd}$ is equal to ${}_dE_{pd}$ in this condition. In the experiment, a gray film with $T_f=0.14$ was used. This means that $R_1R_sT_f^2$ is equal to 1 per cent of R_s, since R_1 is approximately 0.5. Therefore, we can observe the absolute value of ${}_dE_{pd}$ within an error of 0.01 in E units, even when the reflectance of the sample is 100 per cent.

A rather great difference between ${}_dE'_{pd}$ and ${}_dE_{pd}$ is observed in the spectra of a variegated white ivy leaf. Curve A in Fig. 6 is the ${}_dE'_{pd}$ spectrum uncorrected for multiple reflection and Curve B is the ${}_dE_{pd}$ spectrum corrected for it. Although their attenuance values are different, the heights of the bands from their base lines and also the definition and positions of the bands in the two spectra are almost the same. Other examples of corrected ${}_dE_{pd}$ spectra are shown in Figs. 7 and 8. Curve B in Fig. 7 is the spectrum of a leaf of spinach (*Spinacia oleracia*). The spectrum shows the clear absorption bands of chlorophyll *a* and a shoulder due to carotenoids. The position (676 mμ) of the red peak of chlorophyll *a* agrees

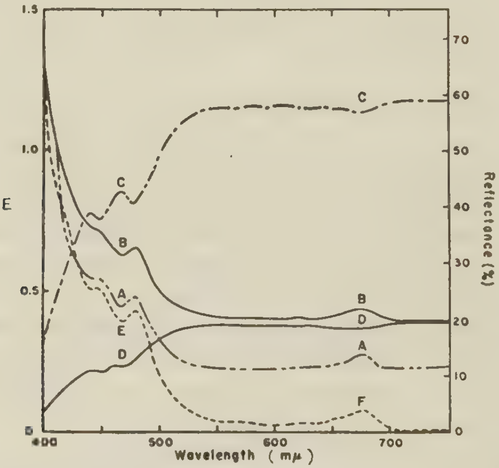

FIG. 6. The relation between the reflectance and transmission spectra of a white ivy leaf.

Curve A; ${}_dE'_{pd}$ spectrum uncorrected for multiple reflection. Curve B; ${}_dE_{pd}$ spectrum corrected for multiple reflection. Curve C; R_s spectrum. Curve D; $\log(1-R_s)^{-1}$ spectrum. Curve F; ${}_{dr}E_{pd}$ spectrum. The scale on the right is for Curve C.

well with that found in the uncorrected ${}_dE'_{pd}$ spectrum of a bean leaf previously observed (*1*). Curve B in Fig. 8 is the spectrum of a dark red leaf of *Coleus blumei* var. *Verschaffeltii* (variegated form), which has an absorption band due to anthocyanin at 541 mμ.

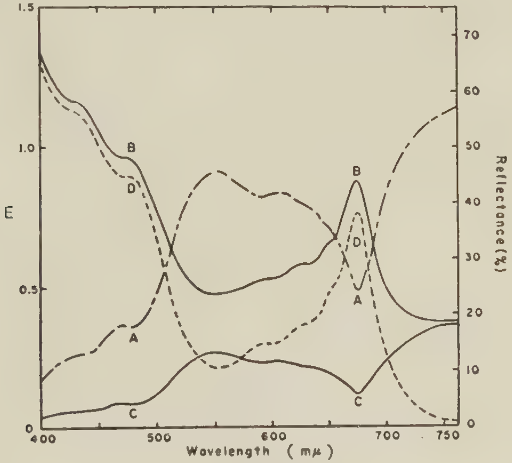

FIG. 7. The spectra of a spinach leaf.
Curves A, B, C and D are R_s, ${}_dE_{pd}$, $\log(1-R_s)^{-1}$ and ${}_{dr}E_{pd}$ spectra, respectively. The scale on the right is for Curve A.

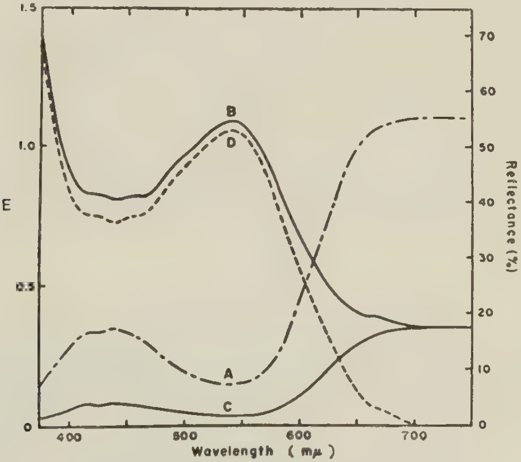

FIG. 8. The spectra of a coleus leaf.
Curves A, B, C and D and R_s, ${}_dE_{pd}$, $\log(1-R_s)^{-1}$ and ${}_{dr}E_{pd}$ respectively. The scale on the right is for Curve A.

By the same principle as before, we can determine the absolute value (${}_pE_{pd}$) of semi-integral attenuance with parallel-light illumination, using gray film and opal glasses. In this case again, only the base line of the uncorrected ${}_pE'_{pd}$ spectrum is raised slightly and no sharpening and shifting of the bands occur by the correction for multiple reflection. Therefore, for practical purposes of observing clear absorption bands, the observation of the uncorrected ${}_pE'_{pd}$ spectrum with no gray film is the best among the observations of four semi-integral attenuances for parallel and diffuse incident lights, with and without the correction. Namely, the base line of the uncorrected ${}_pE'_{pd}$ spectrum is the lowest and we can use the the narrowest slit width with no gray film, thus giving a wider usable wavelength range. However, in order to obtain the absolute value of semi-integral attenuance particularly of highly light-reflecting samples, a correction has to be included for the effect of multiple reflection.

III. Use of Rectilinear Attenuance for the Determination of Cell Concentration

Rectilinear attenuance defined by equation (4), can be used for the measurement of the concentration of suspended particles or cells. If n is the number of cells per ml. of a suspension of a microorganism and the effective and average cross-sectional area of cells is S, the rectilinear attenuance of the suspension can be expressed as a function of these quantities:

$$ {}_pE_p = dnS \qquad (9) $$

where d is the thickness of the container. Therefore, dividing the observed value of ${}_pE_p$ by d, we can obtain nS. The value of nS is a measure of the concentration of the suspension, although the dimensions are different from that of n or packed cell volume, which has been used as the measure of concentratian of cell suspensions. The method of measuring ${}_pE_p$ is superior to the nephelometric method of measuring scattered light, because the ${}_pE_p$ measurement directly gives us the absolute value of nS, while nephelometric observation is based upon an empirical formula, which depends on the apparatus and the microorganisms to be measured. The experimental technique for the observation of ${}_pE_p$ and the data on various microorganisms will be reported in a separate paper.

IV. Principles of the Opal Glass Reflection Method

The difference between reflected and diffuse transmitted light is a matter of angle of deflection, so that we can expect clear bands in the reflection spectra of biological samples. Rabideau, French and Holt (*8*) have succeeded in observing sharp bands in the reflectance spectra of plant materials with an integrating sphere, and Jacquez, Kuppenheim, Dimitroff, McKeehan and Huss (*9–14*) have developed the theory and observed also clear reflectance spectra of the skins of animals. Several techniques with an integrating sphere, which have been proposed so far, are reviewed by French and Young. (*15*). The principle of the opal glass reflection method (*7*) is different from that of the integrating sphere.

Before explaining the principle of the opal glass reflection method, let us illustrate first the apparatus we used for the measurement. A Beckman DK-2 Recording Spectrophotometer was used with an attachment which was set in place of the cell holder usually used for the observation of absorbance of solutions. The side view of the half of the attachement for the sample beam is shown in Fig. 9, where the path of light is indicated by arrows, The other half for the reference beam is similar, but no sample (s) is placed behind the opal glass (g). The light beam is raised by two prisms (p) and goes through a small window (w_1), which cuts down the beam size to a suitable cross section. After passing through the window, the light is reflected back by a front surface mirror (m) to a sheet of opal glass (g). The opal glass is the same kind as for the opal glass transmission method. It has an opalescent coating only on one side of the surface. The angle of the incident beam to the normal to the plane of the opal glass was 32°. Behind the opal plate, the sample or another opal plate was set with a block behind it covered with black velour paper. The diffusely reflected light from the opal glass was detected by the photomultiplier (d) in spectrophotometer through a window (w_2). With a sheet of opal glass in each beam, and no sample, we obtain an almost constant reading against wavelength.

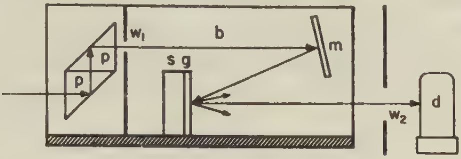

FIG. 9. Attachment for absolute reflectance measurement.

Fig. 5 shows the amounts of the lights reflected from the system on the sample side with a sheet of opal glass and a light-reflecting sample superimposed. As is clear from this figure, the amount of total reflected light (I_{1+s}) is as follows;

$$I_{1+s}=I_0R_1'+\frac{T_1'T_1R_s}{1-R_1R_s} \qquad (10)$$

if there is another sheet of identical opal plate in place of the sample, the amount of total reflected light (I_2) from these two opal plates is expressed in the same way.

$$I_2=I_0R_1'+\frac{T_1'T_1R_1}{1-R_1^2} \qquad (11)$$

Since the amount of the total reflected light (I_1) from a sheet of opal plate is I_0R_1', the difference between I_1 and I_{1+s} or I_2 is

$$D_{1+s}=I_{1+s}-I_1=\frac{I_0T_1'T_1R_s}{1-R_1R_s} \qquad (12)$$

$$D_2=I_2-I_1=\frac{I_0T_1'T_1R_1}{1-R_1^2} \qquad (13)$$

where D_{1+s} and D_2 are those differences, respectively. Therefore,

$$\frac{1}{R_s}=R_1+\frac{D_2}{D_{1+s}}\left[\frac{1}{R_1}+R_1\right] \tag{14}$$

From this equation, we can calculate R_s, the absolute value of reflectance of the sample, if we know the values of D_2/D_{1+s} and the reflectance of an opal plate, R_1 for diffuse light.

The observalion of R_1 of an opal plate was carried out, with the same principle as for the correction for the multiple reflection. The ratio of the transmittance (T_2) of two opal plates to that (T_{2+f}) of two opal plates and a sheet of gray film inserted between them becomes as follows;

$$\frac{T_2}{T_{2+f}}=\frac{1-R_1^2T_f^2}{(1-R_1^2)T_f} \tag{15}$$

since the above ratio is directly observable and T_f can be measured by the opal glass transmission method, we can calculate the value of R_1 by equation (15). The values of R_1 of an opal plate used for this study in the range between 380 and 750 mμ are listed in Table. I.

TABLE I

The Difluse Spectral Reflectance, R_1 of an Opal Glass

Wavelength ($m\mu$)	R_1	log ($1/R_1$)
750	0.451	0.346
700	0.459	0.338
650	0.468	0.329
600	0.476	0.322
550	0.485	0.314
500	0.493	0.307
450	0.500	0.301
400	0.504	0.297
380	0.499	0.302

Once R_1 of an opal plate is measured against wavelength, the only observation neccessary for each sample is the measurement of D_2/D_{1+s}. For the observation of this ratio, the electronic recording system of the spectrophotometer was set so as to record the ratio of the intensity of the sample beam to that of the reference beam. Therefore, the relative values of I_1, I_2 and I_{l+s} were recorded with a sheet of opal glass, two sheets superimposed, and a sheet of opal glass with the sample, respectively. A sheet of opal plate was always placed on the reference side for these measurements. Since we need only the ratio of D_2 to D_{1+s}, we can calculate the value from the relative values of I_1, I_2 and I_{1+s}. This feature of the observation makes it possible to expand the scale or to shift the zero level electronically, depending upon the sample. The value of diffuse reflectance of MgO observed by this method agreed fairly well with the values observed by other methods (*7*)

V. Reflection Spectra of Leaves in Relation to Their Transmission Spectra

Leaves were chosen to illustrate the relation between reflection and transmission spectra for the following reasons. i) Leaves have rather high reflectance in the spectral range where there is no absorption. The condition is favorable for obtaining the relationship. ii) Leaves are thin enough to eliminate the possibility that some light may be lost from the side of the sample without being measured by either transmission or reflection measurement. iii) They require no container.

The reflectance spectrum of the same white ivy leaf as for the measurement of the ${}_dE_{pd}$ spectrum was observed by the opal glass reflection method. The spectrum in R_s units is shown in Curve C in Fig. 6, where the scale of R_s is indicated on the right side of the figure. Now, we have the absolute values of the reflectance and the semi-integral attenuance of the same leaf. To correlate these values, a new quantity, *R-corrected* (Reflection corrected) *attenuance* was defined; that is the logarithm of the ratio of I_0-I_r to I_t. The difference between R-corrected and semi-integral attenuances is in the reference light. In R-corrected attenuance, the amount of the light, which actually goes into the sample without being reflected, was taken as the reference quantity. Therefore, R-corrected attenuance depends only upon the transmitting property of a sample, without being affected by the reflecting property. In other words, it is a sort of idealized quantity of semi-integral attenuance, which would be obtained from an idealized sample that does not reflect the incident light at all but nevertheless transmits the light in the same way as an actual sample does. R-corrected attenuance for parallel and diffuse incident light will be expressed by ${}_{pr}E_{pd}$ and ${}_{dr}E_{pd}$, respectively.

For diffuse incident light, we obtain the following equation;

$$\begin{aligned} {}_{dr}E_{pd} &= \log\frac{I_0-I_r}{I_t} = \log\frac{I_0}{I_t} + \log\frac{I_0-I_r}{I_0} \\ &= \log(I_0/I_t) + \log(1-R_s) = {}_dE_{pd} - \log(1-R_s)^{-1} \qquad (16) \end{aligned}$$

Equation (16) indicates that the value of ${}_{dr}E_{pd}$ can be obtained by subtracting $\log(1-R_s)^{-1}$ from ${}_dE_{pd}$. The spectrum of $\log(1-R_s)^{-1}$ of the white ivy leaf was calculated from Curve C in Fig 6 and is shown as Curve D in the figure. The comparison of Curves B ane D shows clearly that these curves match where there is no absorption band beyond 700 mμ. As a consequence, the base line of the ${}_{dr}E_{pd}$ spectrum (Curve F) coincides with the zero line. This fact proves that the existence of a certain height of base line in the ${}_dE_{pd}$ spectrum is due to the reflection of the sample. It also proves that the values of R_s and ${}_dE_{pd}$ observed by opal glass techniques were exact.

Other examples of the ${}_{dr}E_{pd}$ spectra of spinach and *Coleus* leaves are shown in Figs. 7 and 8, where Curves A, B, C and D are their R_s, ${}_dE_{pd}$, $\log(1-R_s)^{-1}$ and ${}_{dr}E_{pd}$ spectra, respectively. The ${}_dE_{pd}$ and $\log(1-R_s)^{-1}$ spectra in these figures match again where there is no absorption. The positions of the minima of the R_s spectra and the maxima of the ${}_{dr}E_{pd}$ spectra were found to be the same as the maxima of the corresponding bands in the ${}_{d}E_{pd}$ spectra. Therefore, the observation of a reflectance spectrum serves as the observation of a semi-integral attenuance spectrum, so far as the positions of the bands are concerned.

Using the data of the *Coleus* leaf, the relation between the transmittance and the reflectance was obtained. In Fig. 10, the reflectance was plotted against the transmittance calculated from the semi-integral attenuance. While points in this figure were obtained for wavelengths on both sides of the anthocyanin band, they fall on a smooth and slightly bent curve. Other plots of data from different leaves also show curves of a similar type but differently located. Since the bending is small, we can approximately substitute for this curve a straight line through the origin, which is indicated as a ditted line in Fig. 10. Namely, the ratio of R_s to T_s can be assumed to be approximately constant for each sample;

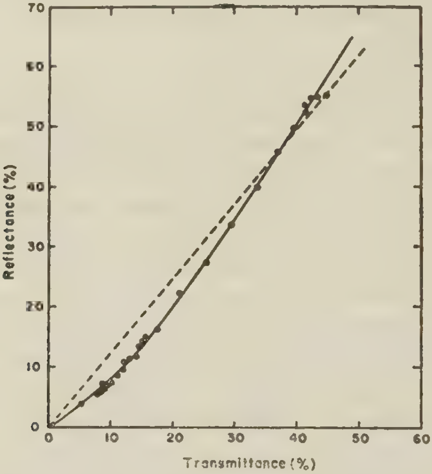

FIG. 10. The relation between the transmittance and reflectance of a coleus leaf.

$$R_s/T_s=\beta \tag{17}$$

where β is a proportionality constant.

Recently, Duysens (*16*) reported a theory on the flattening effect in the spectra of cell suspensions. In order to estimate the amount of the effect, the height of a band *in vivo* in terms of R-corrected attenuance has to be known. If we can approximately use equation (17), the R-corrected attenuance spectrum of a sample can be obtained from the spectrum of semi-integral attenuance only, without measuring the reflectance. Namely, the height of the base line in the ${}_{d}E_{pd}$ spectrum is equal to both $\log(1-R_s)^{-1}$ and $\log(1/T_s)$, where there is no absorption band. From those values, the proportionality constant β in equantion (17) for the sample can be calculated. Therefore, we can calculate the value of $\log(1-R_s)^{-1}$ from the value of $\log(1/T_s)$ at any wavelength, so that we obtain the spectrum of R-corrected attenuance. The sizes of pigment particles *in vivo* evaluated from the flattening effect thus calculated will be reported separately.

VI. Integral Attenuance Spectra of Leaves

Integral attenuanee (${}_dE_{rpd}$) with diffuse-light illumination, which is defined in equation (18), corresponds to the absorbance of transparent materials, in that we can calculate, if we know the amount of I_0, the absolute amount of absorbed light from it.

$${}_dE_{rpd}=\log\frac{I_0}{I_r+I_t}={}_dE_{pd}-\log\left(1+\frac{R_s}{T_s}\right) \qquad (18)$$

The integral attenuance (${}_pE_{rpd}$) for parallel incident light can be expressed in the same way. By equation (18), we can calculate the exact value of integral attenuance from the amunt of ${}_dE_{pd}$, T_s and R_s obtained from semi-integral attenuance and reflectance measurements.

Here again, we can simplify the procedure of obtaining an integral attenuance spectrum of the sample, for which equation (17) holds. Namely, the second term, $\log(1+R_s/T_s)$ is equal to the height of the base line of the semi-integral attenuance spectrum. Since the value of R_s/T_s can be considered to be independent of wavelengths for the sample, the integral attenuance spectrum can be obtained simply by subtracting the base line from the semi-integral attenuance spectrum. Probably this procedure has been used with no theoretical consideration in many studies for estimating the heights of the bands in the spectra of translucent materials. It is now clear, however, that it is the way to obtain integral attenuance spectra and is applicable only to the samples in which the value of R_s/T_s, is independent of wavelength.

VII. Relative Reflectance Spectra of Biological Materials

For obtaining the absolute value of reflectance by the opal glass reflection method, we need some calculations based on directly observed values. Furthermore, if the reflectance of a sample is low in the range of light absorption, it is hard to locate the absorption maximum clearly because of the small change of the reflectance with wavelength. In order to obtain sharp bands directly, even of low-reflecting samples, a way of recording the logarithm (${}_dE_r'$) of the reciprocal of the relative value of reflectance was devised. The logarithm (${}_dE_r$) of the reciprocal of the absolute value was designated as *reflex attenuance*;

$${}_dE_r=\log(I_0/I_r)=\log(1/R_s) \qquad (19)$$

therefore, ${}_dE_r'$ is

$${}_dE_r'=\log(1/R_s')={}_dE_r+C \qquad (20)$$

where R_s' is the relative value of R_s and C is a constant which is independent of wavelength.

The same attachment unit as for absolute reflectance measurement was used but in a different way. The spectrophotometer (Beckman DK-2) was set so as to record the logarithm of the ratio of the intensity of the reference light to that of the sample light. A reference curve was first recorded with an opal plate for both sample and reference sides of the attachment. The result gives us a sligtly sloping but almost straight line. When we observe a sample, it is placed alone, not together with an opal plate, on the sample side. In this case, the incident parallel light from the surface mirror (m) directly strikes the sample at a 32° angle to the normal to the plane of the sample. The specularly reflected light from the sample will hit the bottom of the attachment without being measured. Hence, the light which is reflected back to the detector is diffusely reflected light. If the intensity at the detector of the light from a sample is proportional to the absolute values of R_s, the increment of the reading with a sample above the reference curve is

$$\log(1/R_s')-\log(1/R_1)={}_dE_r+C-\log(1/R_1) \tag{21}$$

It should be remarked, however, that such a proportionality independent of wavelength can not be generally expected from this optical arragement. The values of C obtained from the observed increments may or may not be independent of wavelength.

TABLE II

The Values of R_s' and R_s of a Coleus Leaf

Wavelength ($m\mu$)	R_s	P_s'	R_s'/R_s
700	0.552	0.294	0.533
690	0.546	0.292	0.535
680	0.538	0.290	0.539
670	0.529	0.286	0.541
660	0.510	0.279	0.547
650	0.486	0.264	0.543
640	0.442	0.242	0.548
630	0.390	0.212	0.544
620	0.327	0.178	0.544
610	0.262	0.145	0.554
600	0.207	0.113	0.546
590	0.157	0.087	0.554
580	0.120	0.063	0.525
570	0.090	0.046	0.511
560	0.082	0.036	0.439
550	0.079	0.031	0.392

The leaf of *Coleus* used for the absolute reflectance measurement was observed by this modified method. The observed values of R_s' and R_s are listed in Table II. The values of R_s' in this table were calculated from the observed increaments described above and the values of $\log(1/R_1)$ in Table I. The constancy of the ratios, R_s'/R_s listed in the last column shows that the observed values of R_s' are truly relative values of R_s. The change of the ratio near 550 mμ seems to be due to the error in the observation of the small values of R_s and some non-uniformity of the sample, which might have been placed in slightly different positions in the two experiments. With more experiments with different samples, it was found that the observed value of R_s' is a relative value of R_s, insofar as the sample is a highly light-diffusing material. Almost all biological non-transparent samples diffuse light sufficiently to observe the relative value of R_s by this method, but translucent materials with high transmittance do not diffuse light enough for applying this method. However, for the samples of the latter kind, the opal glass transmission method can be used effectively for obtaining clear absorption bands.

For practical purposes, we need not even correct for the change of $\log(1/R_1)$ with wavelength, since it is small compared with the large change of $\log(1/R_s')$ near the range of light absorption. The recorded spectrum itself with no correction for the change of $\log(1/R_1)$ shows clear bands, from

TABLE III

The Positions of the Bands in the ${}_dE_r'$ Spectra of Biological Materials

The position of an obscure band or a hump is shown in parentheses

Figure and curve	Sample	Position of band (mμ)
11A	Pink petal of sweet pea	531
11B	Violet petal of sweet pea	568
11C	Root of red radish	532
11D	Petal of hellebore	435,533,676
11F	Flowers of ceanothus	(490),530,574,624,(675)
12A	Cake of yeast	412,(525),(558),(595),(640)
12B	Suspension of yeast with ascorbic acid	417,(445),(475),520,548,(556), 600
13A	Inner seed coat of squash	407,(488),(544),589,648
13B	Endosperm of pole bean	407
13C	"	407,(422)
13D	"	407,422,(446),478
14A	Crystal of β-carotene	430,460,486,528
14B	Benzene solution of β-carotene	435,463,492
14C	Root of carrot	428,452,482,515
15A	Crystal of lycopene	(460),484,(515),(570)
15B	Flesh of watermelon	407,435,(457),483,517,562
15C	Benzene extract of watermelon	(430),457,483,517
15D	Ethanol extract of watermelon	400,427

which we can estimate the exact positions of the bands. The spectra, which will be shown in this section are not corrected for the change and also the zero line for each spectrum was chosen arbitrarily. The positions of the main peaks identified in these spectra are shown in Table III.

The most useful application of this technique is the observation of the spectra of samples which can hardly be observed by the transmission method. Those are non-transparent materials or translucent materials with low transmittance. The $_dE_r'$ spectra of various petals are shown in Fig. 11.

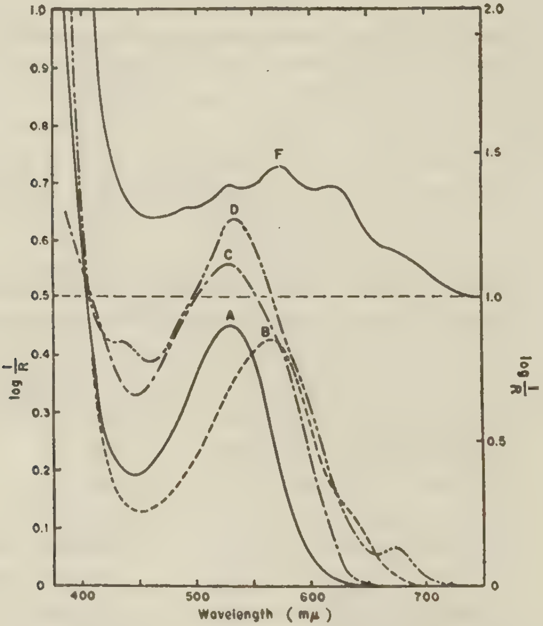

FIG. 11. The $_dE_r'$ spectra of a pink petal of sweet pea (Curve A), a violet petal of sweet pea (Curve B), a root of red radish (Curve C), a petal of hellebore (Curve D) and flowers of ceanothus (Curve F). The scale on the right is for Curve C. The base line for Curve F was raised arbitrarily to avoid confusion.

Curves A and B represent the spectra of the petals of pink and violet flowers of *Lathyrus odoratus* (sweet pea). These petals show distinctly different absorption spectra with maxima at 531 and 568 mμ. The spectrum (Curve C) of the surface of the red root of *Raphanus sativus* (red radish) seems to indicate the presence of the same kind of pigment (532 mμ) as that of the pink petal of sweet pea, with a small amount of an additional pigment, which appears as a shoulder at the longer wavelength side of the main band. In the spectrum (Curve D) of the petal of *Helleborus orientalis* (hellebore), we can see the presence of chlorophyll *a* in addition to an anthocyanin. The spectrum of the flowers of *Ceanothus* (California "Lilac") has three main bands (Curve F). An ethanol extract from the flowers showed a similar absorption spectrum. The curve is symmetrical in shape and resemble the absorption spectrum curve of a carotenoid. But, a carotenoid spectrum which is located at such a long wavelength has not yet been found. Chemical studies may lead to the finding of a new pigment in *Ceanothus*.

Curve A in Fig. 12 shows the $_dE_r'$ spectrum of a cake of commercial Fleishman's yeast. The bands of oxidized cytochromes are seen in the spectrum. The yeast was suspened in water, 16.5 g. wet weight per 100 ml. with a small amount of ascorbic acid. The spectrum (Curve B) shows the bands of reduced cytochromes. The soret band in the reduced form was found to be at 417 mμ instead of at 412 mμ as in the oxidized form.

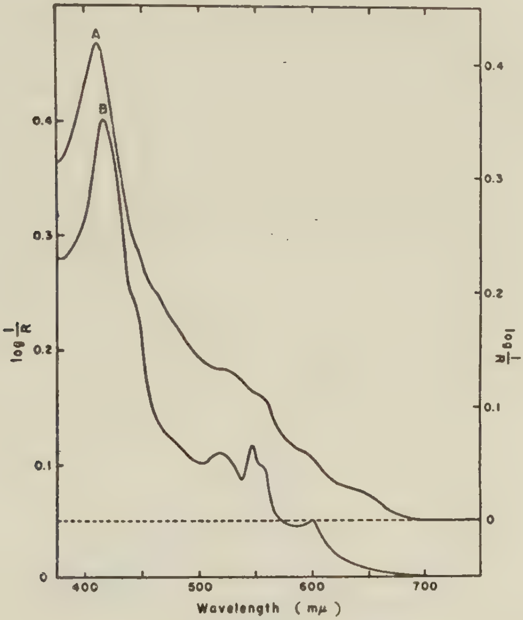

FIG. 12. The $_dE_r'$ spectra of a cake of yeast (Curve A) and a suspension of yeast with ascorbic acid added (Curve B). The scale on the right is for Curve A.

The $_dE_r'$ spectra of seeds are shown in Fig 13. The seed of *Cucurbita pepo* (squash) has a thin and brownish-green inner seed coat. The spectrum (Curve A) of the intact inner seed coat was observed without peeling the coat. The red band at 648 mμ seems to consist of two bands with a shoulder at the shorter wavelength side of the band. Two similar components have been found in etiolated leaves (*2*), in which the corresponding bands were identified at 650 and 636 mμ *in vivo* and the band at 650 mμ was due to protochlorophyll *a*. According to Koski, French and Smith, (*17*), the action spectrum of the transformation of protochlorophyll *a* to chlorophyll *a* has bands at 650, 593, 550 and 445 mμ. These studies indicate that the band at 648 mμ is due to protochlorophyll *a*. The small shift toward shorter wavelength may arise from the overlapping effect of the additional band at 636 mμ. The other bands at 585 and 545 mμ and a small hump at 445 mμ on Curve A seem to be also due to protochlorophyll *a* (*2*). High bands at 407 and 485 mμ are probably due to cytochromes and carotenoids, respectively.

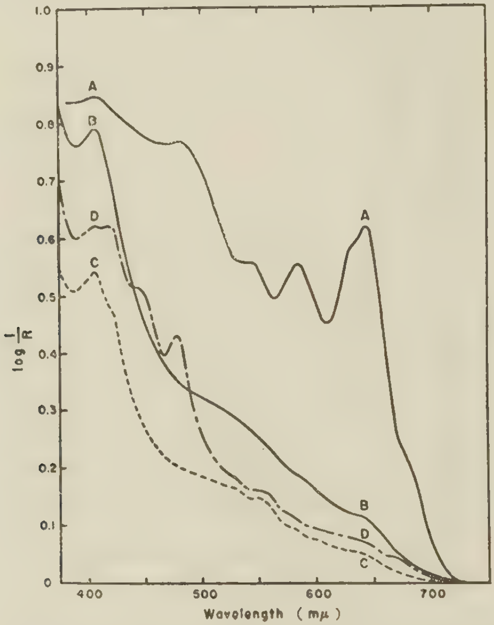

FIG. 13. The $_dE_r'$ spectra of seeds; inner seed coat of squash (Curve A), endosperm of pole bean in the original dry state of the seed (Curve B), endosperm of pole bean after 24 hours in water (curve C) and cotyledon of pole bean plant cultured for ten days in the dark (Curve D)

Curve B in Fig 13 shows the $_dE_r'$ spectrum of the inside surface of the endosperm of *Phaseolus vulgaris* var. Ferry-Morse's Pole bean, No. 191. It was observed in the dry form of the endosperm, which has a sharp band at 407 mμ. The seeds of the pole bean were placed on a wet paper for 24 hours and the $_dE_r'$ spectrum of the endosperm was observed again (Curve C). In this spectrum, we can see a band developed around 422 mμ. This band is, therefore, due to the initial pigment formed in the germination process. Similar seeds were cultured in the dark under soil. The etiolated leaves came out from the cotyledons after a week. After ten days, the cotyledon was picked from the plant and the $_dE_r'$ spectrum of the inner surface was observed (Curve D). The spectrum shows the increase of the band at 422 mμ, and also new bands at 446 and 478 mμ, which are probably due to some carotenoids formed in the culturing process. But, no protochlorophyll band was observed in the red region. This indicates that protochlorophyll *a* is formed only in the embryo of the leaf. Similar studies with different kinds of bean seeds also showed the similar process as that in the bean No. 191.

As a separate experiment, Shibata (*3*) observed the absorption spectra of suspensions of crystalline carotenes and compared them with their spectra in solution. It was found that there is an additional band in the spectrum of the suspension besides the bands which correspond to the bands in solution. This additional band exists at the longer wavelength side of the other bands. It was also found that the position of this band depends upon the size of the crystals in the suspension much more than do the positions of the other bands. The observed spectra of β-carotene are reproduced in Curves A and B in Fig. 14. From the shape of those curves, it is clear that the band at 528 mμ is the additional one.

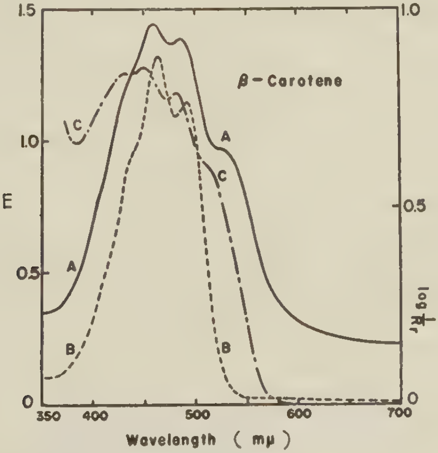

FIG. 14. Spectrum of β-carotene.

Curve A; $_pE'_{pd}$ spectrum of a suspension of crystalline β-carotene, Curve B; absorbance spectrum of the benzene solution, Curve C; $_dE'_r$ spectrum of a root of carrot.

Many investigations have been done on the state of carotenoids *in vivo*, since the earliest observation by Schimper (*18*) until the most recent studies by Straus (*19*). The results were reviewed by Straus (*20*). These studies showed that, in their natural state, some carotenoids may exist as crystals. However, these results were obtained mainly by microscopic observations, which seem to need support from other kinds of observations for the conclusive solution of the problem. With the hope that the additional band found in the suspension of the crystals of β-carotene may found in the spectrum of some intact biological materials, the $_dE_r'$ spectrum of the root of *Daucus carota* var. sativa (carrot) was observed. The spectrum (Curve C in Fig. 14) shows the additional band at 515 mμ. The deviation of the position from the value, 528 mμ, previously obtained in the suspension of the crystals is probably due to the difference in size of the crystals.

Similar experiments were carried out on the state of lycopene *in vivo*. In the experiment with β-carotene (*3*), the suspension was prepared by sonic treatment of the crystals in water with a drop of a soap solution. A different method was adopted for making fine crystals of lycopene. A petroleum ether solution of lycopene was evaporated drop by drop in a small area on a filter paper (Whatman No. 2), in order to obtain fine crystals on the paper. This sample was used for the semi-integral attenuance measurement using a plain filter paper as the reference (*1*). Curve A in Fig. 15 shows the spectrum, which exhibits an additional band at 570 mμ. Curve B in the figure indicates the $_dE_r'$ spectrum of the red flesh of *Cucurbita pepo*, watermelon. Curves C and D are the absorption spectra of the benzene and alcohol extracts from the watermelon, respectively. The comparison of these curves shows that the band at 562 mμ on Curve B corresponds to the characteristic band of lycopene crystals at 570 mμ. A similar band was found at 560 mμ also in the $_dE_r'$ spectra of the fruits of *Lycopersicon esculentum* (tomato) and pink

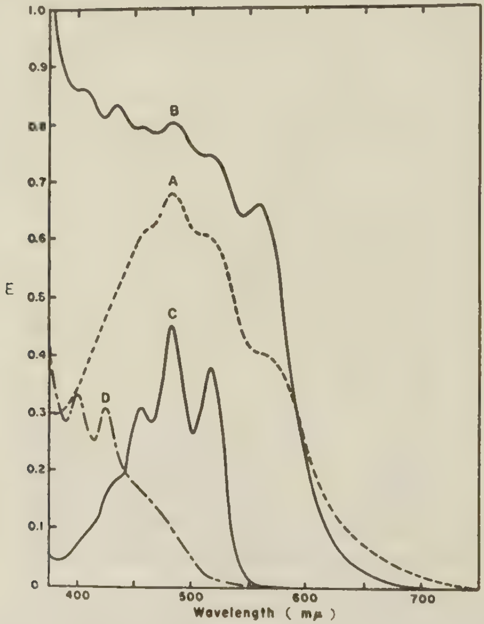

FIG. 15. Spectra of lycopene.
Curve A; $_pE'_{pd}$ spectrum of crystalline lycopene on filter paper, Curve B, $_dE_r'$ spectrum of flesh of watermelon, Curves C and D, absorbance spectra of benzene and ethanol extracts from watermelon.

fleshed variety of *Citrus maxima* (grape fruits). These spectroschpic observations support the point of view that some carotenoids exist in a crystalline form in their natural states. The additional band of carotenoids was observed only in the spectrum of fruits or roots, but not in leaves, petals and microorganisms examined.

DISCUSSION

The names, symbols and definitions of attenuances required for the spectroscopic measurements of translucent or non-transparent materials are summarized in Table IV. It will be worth while to stress the importance of defining these attenuances, since in many spectroscopic studies of translucent biological materials, the word absorbance (optical density) has been carelessly used. Without any detailed description of the optical arragement used or any comment on the definition, it could be any attenuance except R-corrected and reflex attenuance listed in Table IV. At least six attenuances listed in the table are required for avoiding such a confusion. Various spectroscopic observations on biological samples with this new point of view will solve many problems without leaving any ambiguity as to the observed values of attenuances.

TABLE VI

Names, Symbols and Definitions of Attenuanccs

Name	Nature of incident light	
	Parallel light	Diffuse light
Rectilinear attenuance	${}_pE_p=\log\frac{I_0}{I_p}$	—
Quasi-attenuance	${}_pE_{pfd}=\log\frac{I_0}{I_p+fI_d}$	—
Semi-integral attenuance	${}_pE_{pd}=\log\frac{I_0}{I_p+I_d}$	${}_dE_{pd}=\log\frac{I_0}{I_t}$
R-corrected attenuance	${}_{pr}E_{pd}=\log\frac{I_0-I_r}{I_p+I_d}$	${}_{dr}E_{pd}=\log\frac{I_0-I_r}{I_t}$
Integral attenuance	${}_pE_{rpd}=\log\frac{I_0}{I_r+I_p+I_d}$	${}_dE_{rpd}=\log\frac{I_0}{I_r+I_t}$
Reflex attenuance	${}_pE_r=\log\frac{I_0}{I_r}$	${}_dE_r=\log\frac{I_0}{I_r}$

I_0—amount of incident light, I_t—amount of total transmitted light, I_p—amount of parallel transmitted light, I_d—amount of diffuse transmitted light, I_r—amount of reflected light, I_a—amount of absorbed light.

In this report, the theories and techniques of opal glass transmission and reflection methods were described. From both experimental and theoretical points of view, the opal glass methods for absolute measurement are certainly simple, compared with the classical use of an integrating sphere. Probably the only question left about opal glass methods would be whether an opal plate is a complete light diffuser or not. However, if the effect of this factor were serious, we would not have obtained such good agreement between the values of semi-integral attenuance and $\log(1-R_s)^{-1}$ of leaves, in the range where there was no absorption. The observations (*7*) on the reflectance of MgO also support the conclusion that the effect is within the experimental error even if it exists. Of course, the angular distribution of the intensity of the light transmitted through the opal plate will tell how complete the diffusing property is.

For the practical purpose of obtaining clear absorption bands, the opal glass transmission method previously proposed (*1*) and the modified opal glass reflection method for observing $\log(1/R_s')$ will be most useful. For translucent materials, the transmission method can be applied. If a sample is non-transparent or low light-transmitting, the reflection method can be used. Therefore, we can observe the bands of any biological sample clearly and simply, regardless of the optical property of the sample. These methods for relative measurement are even simpler and will be more useful in many biological researches than the opal glass methods for absolute measurement. However, the principles of the methods for relative measurement are based upon the theories of opal glass methods for absolute measurement and the experimental results presented in this report.

SUMMARY

1. The theories and techniques of measuring the absolute values of the transmittance and reflectance of translucent biological materials with opal glass plates are presented. These methods were called *opal glass transmission* and *reflection methods*. They are simple and easy to practice with spectrophotometers commonly used for the measurement of transparent materials. From the transmission and reflection spectra of leaves observed by these methods, it was proved that they give us the exact values of the transmittance and reflectance.

2. From the results of the measurements by opal glass transmission and reflection methods, it was found that we need to define at least six quantities, which describe the optical properties of translucent or non-transparent biological materials. The way and use of evaluating these properties are described, especially paying attention to the absolute measurement of the light absorbed by those samples.

3. By modifying the opal glass reflection method, we can observe the logarithm of the reciprocal of the relative value of reflectance, even more simply than by opal glass reflection method for absolute measurement. The measurements by the modified method showed clear absorption bands of biological non-transparent materials. As one of the application of the method, the state of carotenoids *in vivo* was studied. The results indicated that some carotenoids exists in their crystalline state in roots or fruits.

The author wishes to express his heartfelt thanks to Dr. C. S. French for his guidance and also his constant encouragement during this investigation. The samples of various seeds and pure lycopene crystals were obtained by the courtesy of Dr. J. H. C. Smith. The author also wishes to thank Dr. H. Tamiya and Dr. H. W. Milner very much for many suggestions in the preparation of this manuscript.

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PREPARATION OF 3α,7α,12α-TRIHYDROXY-24α-OXOCOPROSTANE AND 3α,7α,12α,24ξ-TETRAHYDROXYCOPROSTANE

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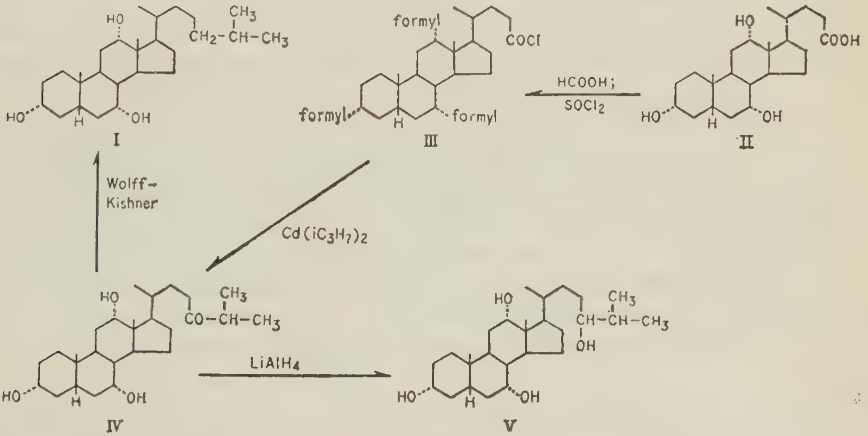
Since the classical study of Bloch, Berg and Rittenberg *(1)* (1943), there have been accumulated evidences *(2)* that the C_{24} bile acids are derived from cholesterol in the dog or rat organism. The metabolic pathway of cholesterol to cholic acid, however, is still largely unknown. It has recently been shown *(3)* that C^{14}-3α,7α,12α-trihydroxycoprostane, injected into the rat organism, was rapidly converted into C^{14}-taurocholate in a good yield, while labeled cholesterol administered was converted at a slower rate into C^{14}-taurocholate.

As to the pathway of the trihydroxycoprostane (I) to cholic acid (II), Haslewood *(4)* proposed that the trihydroxy sterol was oxidized at the methyl group C_{27} to give 3,7,12-trihydroxycoprostanic acid-C_{27}, which, in turn, underwent β-oxidation to afford cholic acid. Komatsubara *(5)* reported that a coprostanic acid isolated from bull frog bile was transformed into cholic acid in the guinea pig organism. With tritium labeled 3α,7α,12α-trihydroxycoprostanic acid Bergström et al. *(6)* have recently shown that the main reaction in rat liver homogenate is its degradation to cholic acid followed by conjugation with taurine.



On the other hand, C_{24}-hydroxy or C_{24}-oxo derivative of 3α,7α,12α-trihydroxycoprostane might be a possible intermediate of the latter to cholic acid in the mammal. We, therefore, have prepared 3α,7α,12α-trihydroxy-24-oxocoprostane (IV) and 3α,7α,12α,24ξ-tetrahydroxycoprostane (V) for the metabolic studies: Condensation of triformyl cholyl chloride (III) with diisopropyl cadmium (*7*) gave 3α,7α,12α-trihydroxy-24-oxocoprostane (IV), reduction of which with lithium aluminum hydride yielded 3α,7α,12α,24ξ-tetrahydroxycoprostane (V) in an excellent yield.

The trihydroxy-24-oxocoprostane was reduced by the Wolff-Kishner reduction (Huang-Minlon (*8*)) to give exactly the same trihydroxycoprostane (I), as had been prepared by the Kolbe procedure (*3*, *9*).

EXPERIMENTAL

3α,7α,12α-Trihydroxy-24-oxocoprostane (IV)—According to Ruzicka et al. (*10*), triformyl cholyl chloride (III) was prepared from 8 g. of triformyl cholic acid. Without being isolated, the chloride was dissolved in dry benzene and poured into a solution of diisopropyl cadmium, which was prepared from 4.9 g. of magnesium, 19 ml. of isopropyl bromide, 200 ml. of absolute ether and 20.3 g. of anhydrous cadmium chloride (*7*).

The reaction mixture was allowed to stand overnight, and then worked up in the usual manner. The reaction product was refluxed for 1/2 hour in methanolic KOH solution, and extracted with ether. The evaporated extract was triturated with warm petroleum ether and yielded 2.0 g. of tiny needles, melting at 138–140°. The crystals were recrystallized three times from isopropyl ether-petroleum ether (b.p. 55–70°). Needles of m.p. 141–142°.

Analysis Calcd. for $C_{27}H_{46}O_4$: C, 74.65; H, 10.60.
Found: C, 74.94; H, 11.03.

Oxime of 3α,7α,12α-trihydroxy-24-oxocoprostane—A mixture of the trihydroxycoprostanone (45 mg.) described above, hydroxylamine hydrochloride (0.1 g.) and sodium acetate (0.2 g.) in methanol (10 ml.) was refluxed for 1.5 hours.

The mixture was diluted with water, the resulting oxime being precipitated and collected. Two recrystallizations from aqueous methanol gave 20 mg. of needles, decomposing at 206–207°

Analysis Calcd. for $C_{27}H_{47}O_4$: C, 72.16; H, 10.47; N, 3.12.
Found: C, 72.18; H, 10.60; N, 3.20.

3α,7α,12α,24ξ-Tetrahydroxycoprostane (V)—A solution of 100 mg. of the trihydroxycoprostanone (IV) in 10 ml. of absolute ether was added, with stirring, to an ethereal solution of lithium aluminum hydride (100 mg. in 10 ml.), and stirred for two hours at room temperature.

The reduction mixture was decomposed with ice-cold dilute sulfuric acid. Ethyl acetate was added and shaken vigorously. The organic phase was separated, washed with dilute sulfuric acid, saturated sodium bicarbonate solution and water, successively, and then dried over Na_2SO_4.

After the solvent was evaporated, the residue was recrystallized three times from ethyl acetate-petroleum ether (b.p. 55–70°), giving 65 mg. of needles. m.p. 173–174°.

Analysis Calcd. for $C_{27}H_{48}O_4$: C, 74.31; H, 11.01.
Found: C, 74.49; H, 11.19.

Reduction of 3α,7α,12α-trihydroxy-24-oxocoprostane to 3α,7α,12α,-trihydroxycoprostane (I)—A mixture of 100 mg. of the trihydroxycoprostanone (IV), 0.5 g. of sodium hydroxide and 0.2 ml. of hydrazine hydrate (85 per cent purity) in 5 ml. of triethylene glycol was refluxed and conducted in the same manner as was described by Huang-Minlon (*8*). The reaction mixture was diluted with water and extracted with ether.

The ether extract was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue was recrystallized twice from ethyl acetate, giving 3α,7α,12α-trihydroxycoprostane melting at 186°, just like the sample prepared by electrolysis (3, 9).

SUMMARY

3α,7α,12α-Trihydroxy-24-oxocoprostane was prepared by treating triformyl cholyl chloride with diisopropyl cadmium. The 24-oxo-compound was subjected to lithium aluminum hydride reduction to give 3α,7α,12α,24ξ-tetrahydroxycoprostane.

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STUDIES ON AMYLASE FORMATION BY BACILLUS SUBTILIS

V. IMMUNOCHEMICAL STUDIES OF AMYLASE PRODUCED BY BACILLUS SUBTILIS

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From the previous studies of this series (*1–5*) it was proposed that the last stage of amylase formation by *Bacillus subtilis* does not involve free amino acid utilization but is a transformation of a preformed complex precursor substance. This conclusion was derived mainly from the results both of inhibition experiments using amino acid analogues (*2*) and of the direct isotopic analysis on the amylase protein produced in the presence of S^{35}-methionine (*5*). The possibility that this proposed precursor material might be immunologically related to amylase protein, prompted us to undertake the immunochemical studies of amylase.

The application of immunochemical methods to the study of enzyme formation was already performed by Cohn and Torrriani (*6*, *7*) in their pioneer work on the β-galactosidase system of *E. coli*, and later, by Pollock (*8*, *9*) and Suskind *et al.* (*10*). The present paper describes the similar approach to problems of amylase formation. The immunochemical aspects of amylase-anti-amylase system will be described in another paper (*11*).

Rabbit serum antibody against partially purified molt amylase preparation had been demonstrated by Lüers and Albrecht. They had showed a neutralization of amylase activity by the serum antibody, and an inhibitory effect of starch for the neutralization of enzyme activity by the antibody. They also showed that amylases from pancreas, and saliva, did not cross-reacted with the antibody against molt amylase. No available information, however, is obtained so far about the antibodies against crystalline amylase proteins.

METHODS

Crystalline amylase (*12*) obtained from the culture medium of *Bacillus subtilis* N was used as an antigen. Amylase activity was assayed according to the method of Hagihara (*12*). One mg. of crystalline amylase used has an activity of 2800 units ($D^{30\,\text{min.}}_{40°}$). Crystalline amylase was dissolved in 0.85 per cent NaCl solution at a concentration of 10 mg./ml., 0.5 ml. of this solution was injected intravenously into rabbits. Two days later each rabbits received five further intravenous injections, 1.0 ml. per injection, on alternate days. The rabbits were bled from carotid artery 12 days after the last injection and the sera were separated, inactivated at 56° for 30 minutes and stored in a frozen state. Amylase activity contained in the serum thus obtained was negligible compared to that bacterial amylase used as an antigen in a test system.

Antiseum was usually diluted with physiological saline (0.85 per cent NaCl). Amylase solution used in a test system was prepared in calcium acetate solution ($M/500$) containing 0.02 per cent of sodium azide.

RESULTS

Neutralization of Enzymatic Activity—The amylase neutralizing power of antiserum was demonstrated by the almost complete abolishment of the activity of crystalline amylase solution incubated with antiserum. Normal control serum inhibited the amylase activity only very slightly and therefore, the inhibition due to normal component in the serum could be neglected. First, the time course of the inhibition of amylase activity by antiserum was studied. The known amount of amylase was incubated with a suitably diluted antiserum. After various incubation periods, the remaining amylase activity was assayed. The neutralization reaction, measured in terms of amylase activity inhibition, was almost complete in 1 hour. Usually incubation time of 1 hour was used in the subsequent experiment.

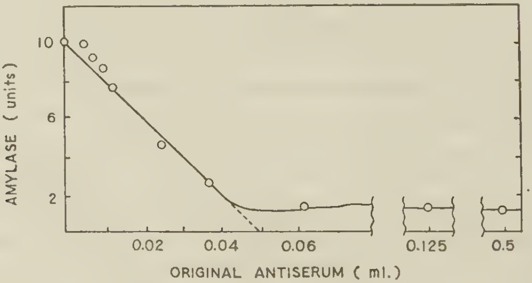

FIG. 1. Neutralization curve of crystalline bacterial amylase with antiserum.

0.5 ml. of amylase solution (10.2 units) was mixed with 0.5 ml. of antiserum solutson containing various amount of original antiserum. Enzyme activity was assayed after 1 hour at 40°

Quantitative relationship between the amount of enzyme neutralized and that of antibody was studied. Varying concentrations of antiserum were added to the constant amount of amylase and incubated at 40° After 1 hour, the enzymatic activity was determined. The results were presented in Fig. 1. The curve was approximately linear until about 90 per cent of the activity was neutralized, that is, the amount of enzyme neutralized was proportional to that of antibody added in the region of antigen excess. This result was quite analogous to that obtained by Pollock in the penicillinase-anti-penicillinase system (*8*) and permitted the assay of anti-amylase neutralization activity of the serum. Thus an anti-amylase neutralization unit (N. U.) was defined as the amount capable of neutralizing 1 unit (about 0.36 μg.) of amylase and assayed from the slope of neutralization curve obtained in the similar way to that described in Fig. 1.

The small amount of residual activity (usually about 10 per cent) remained unneutralized even in the presence of great excess of antibody.

The antiserum obtained against crystallin α-amylase of *Bacillus subtilis* N was found to neutralize the α-amylase of *Bacillus subtilis* H in the quantitatively same way as that of strain N. Thus both amylases seem to be immunochemically identical.

The effect of antiserum on the various amylase of other origins was tested. No inhibitory effect of the antiserum was observed on the following α-amylases: Crystalline Taka amylase A of *Aspergillus oryzae*, partially purified α-amylase of *Aspergillus usami* and purified malt α-amylase.

Search for Other Substance Immunochemically Related to Amylase—As described in the introduction of this paper, the complex material other than amino acid was proposed to exist within the cell as a direct precursor of amylase. In order to examine the possibility that this proposed precursor material might be immunochemically related to amylase protein, search for the substance, which is immunochemically related to amylase but enzymatically inactive, was performed both in cultural media and in cell extracts.

Cells were cultivated in Soy medium. At the various phases of cultural development, when amylase was not yet accumulated significantly in the in the medium, chloramphenicol was added at a final concentration of about 30 mg./ml. to prevent further enzyme production. Cells were separated from the medium by centrifugation, washed once with $M/30$ phosphate buffer (pH 7.2) containg chloramphenicol (10 μg./ml.) and resuspended in the same buffer solution. The final cell suspension was concentrated to about one tenth to one twentieth of the volume of the original culture. The cell were then either lysed with crystalline lysozyme or disintegrated by a sonic oscillator. Samples of the disintegrated cell preparation or of the cultural filtrate were incubated with 0.2 ml. of suitablly diluted antiserum (usually 10 N. U.) at 40° for 1 hour or at 0° overnight. The mixture as such, or its supernatant solution separated from the insoluble material by centrifugation was tested for its neutralization activity. Thus, the reduction of neutralization activity due to the added sample ("serum blocking power" (SBP)) was measured and compared with the amylase activity of the sample. The SBP was expressed as an equivalent amount of amylase which caused the same amount of reduction ("amylase equivalent" (am. eq.)). The accuracy of the method for the determination of SBP was tested using the known amount of amylase. The quite satisfactory results were obtained ((*c. f.* Table I).

The examination of the cultural filtrate obtained from the culture grown in Soy medium revealed that it contained some substances other than amylase capable of blocking anti-amylase activity of serum (Table I). It was first supposed that the reduction of anti-amylase activity might be caused by the cross reaction of the enzymatically inactive protein closely related to amylase.

The isolation of this substance was attempted. However, it was revealed that this substance was not protein, but small molecular weight substance, since the serum blocking power disappeared completely after dialysis (Table II). Subsequent studies clearly established that it is an oligosaccharide produced by the enzymatic hydrolysis of soluble starch contained in large amounts in the orignal Soy medium (Table III). An apparent competition between the antibody and the substrate of enzyme *i. e.*, starch or its hydrolytic products, for the same portion of enzyme surface was suggested and will be described in the subsequent paper (*11*).

TABLE I

Appearence of the Serum Blocking Substance in the Medium

Sample No.	Sample Cultivation (*hr.*)	Amylase activity (*μ./ml.*)	SBP (*am. eq./ml.*)
1S	2.5	1.0	3.2
2S	4	1.5	4.6
3S	5.5	3.8	7.0
4S	7	4.6×4	4.4×4
Amylase 1.7 *μ*g.		4.7	4.9
Amylase 3.4 *μ*g.		9.4	8.4

Cells of *B. subtilis* N were cultivated in LBY medium for 20 hours at 30°. This culture was diluted with 10 volume of Soy medium (0 time) and shaken further. At indicated times, a portion of the culture was withdrawn and chloramphenicol was added. Cells were removed by centrifugation. Amylase activity and SBP were measured as described in the text. SBP of crystalline amylase solution was determined simultaneously.

TABLE II

Attempt to Isolate the Serum Blocking Substance from the Medium

Sample	Amylase activity (*μ./ml.*)	SBP (*am. eq./ml.*)	SBP/Amylase
(1) Original cultuae medium (2S)*	1.5	4.6	3.1
(2) After aceton precipitation (75%) of (1)	4.4	12.3	2.8
(3) After dialysis of (2)	4.3	3.0	0.7

* Sample listed in Table I.

As to the serum blocking power of the cell extracts, results varied. Though cell extracts were prepared after wshing of cells to prevent the

TABLE III

The Apparent Reduction of Anti-Amylase Activity by the Hydrolytic Products of Starch

Sample	Amylase activity (*μ./ml.*)	SBP (*am. eq./ml.*)
Crystalline amylase	4.5	3.5
" "	9.0	8.0
Soy medium incubated with small amount of amylase*.	1.8	4.5
Starch hydrolysate** 6.4%	1.0	6.3
3.2%	"	2.5
0.8%	"	0.8

* Soy medium (coutaining 8 per cent of soluble starch) was incubated with small amount of crystalline amylrse until the negative point of iodine color reaction of starch. 0.2 ml. of this solution was used in the final volume of 1 ml. of test system. Therefore, the concentration of starch hydrolysate in the test system is 1.6 per cent (based on the original amount of starch).

** Sixteen per cent of soluble strach solution was incubated with the small amount of amylase (final concentration of about 1 μ./ml.; Azide (0.01 per cent) was also added as a preservative), until the iodine reaction of the starch became negative. The concentration of hydrolysate was expressed as that in the test system.

TABLE IV

Serum Blocking Power of Disintegrated Cell Preparation

No.	Sample cultivation (*hr.*)	Amylase (*μ./ml.*) in medium at the time of harvestigation	in Sample	SBP (*am. eq./ml.*)
1C	3.5	0.6	0.3	4.3
2C	5	1.5	0.8	3.0
3C	6	6.0	1.8	2.2
4C	8.5		6.9	8.1
Crystallin amylase			9.2	7.0

Cell were cultivated in the similar way to that described in Table I. At indicated times, the cells were harvested and disintegrated by the sonic oscillator as described in the text. Amylase activity both in the sample of disintegrated cell and in the medium at the time of harvestigation was assayed. SBP of the disintegrated cell preparation was then assayed.

contamination of oligosaccharides in the medium, the significant reduction of anti-amylase activity was sometimes demonstrated. Table IV shows one example of positive data. However, the conditions, which assures the accumulation of the serum blocking substance in the cell, could not be established. Furthermore, the SBP in the positive sample disappeared after dialysis, just as in the case of the sample of cultural filtrate. The positive reduction of anti-amylase activity was interpreted to be due to the oligosaccharides taken up from the medium. The question, whether the accumulation of active so called " internal inducer ", was not studied further. At any rate, it was concluded that any protein capable of combining with anti-amylase neutralizing antibody other than amylase was not accumulated in significant quantity either in the medium or in the cell.

The Question of Intracellular Amylase—Pllock has reported that a portion of cell-bound penicillinase of *Bacillus cereus* is immunochemically distinct from exo-penicillinase (*9*). The similar possibility in our case was examined, though the formation of amylase by our bacteria is very different from that in Pollock's system, and the amount of cell-bound amylase is very small compared with that released to culture medium.

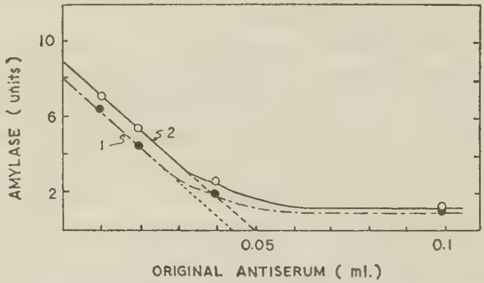

FIG. 2. Neutralization curve of cell bound amylase (Curve 1) and crystalline exo-amylase (Curve 2) with antiserum prepared against exo-amylase.

Experimental condition is similar to that described in Fig. 2.

Cells were grown in Soy medium and cultivated in the phase of linear production of amylase. After washing three times with phosphate buffer, cell were suspanded in small amount of buffer making a very concentrated suspension, disintegrated by sonic oscillation and the cellular debris was removed by centrifugation. Neutralization of amylase activity of this cell extract with antiserum against crystalline exo-amylase was examined and compared with that of crystalline exo-amylase. As shown in Fig. 2, the same neutralization curve was obtained and the cell-bound amylase was concluded to be immunochemically identical with exo-amylase. From the similar examination on the crude cultural filtrate obtained under various conditions, it was concluded that this bacterium produces only one and the same enzyme under all conditions.

Effect of Anti-Amylase Serum on the Formation of Amylase by Intact Cells—It is very interesting to see the effect of antienzyme on the enzyme formation, especially in connection with the role of inducer in the induced formation of enzyme.

The effect of antiserum on the amylase formation by intact cells was examined, through the problem of permeability of antibody through cell surface remained in the intact cell system.

Cells were grown in Soy medium. At the late log phase when the culture had not yet begun to produce amylase, dialyzed antiserum was added to the culture. One control culture received the same amount of dialyzed normal serum, and the other recived the physiological saline. Curltures were aerated further and amylase productions were followed. The result was presented in Fig. 3. Normal serum inhibited the amylase formation greatly. Antiserum caused further reduction of the amount of amylase formed. However, this amount nearly corresponded to that combined by the quantity of antibody used. Thus any effect due to the antibony *per se* could not be demonstrated.

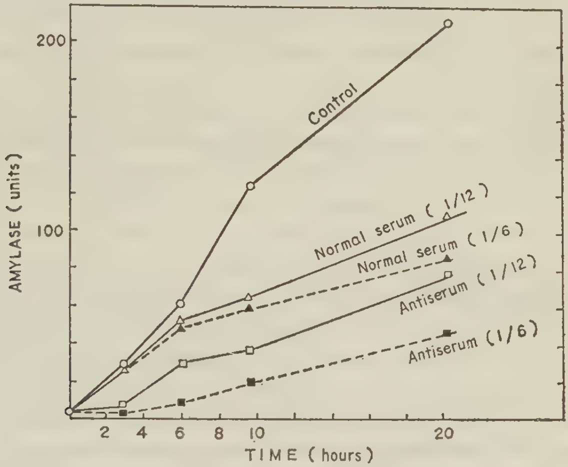

FIG. 3. Effect of anti-amylase serum on the formation of amylase by intact cells.

Cells were cultivated in LBY medium at 30° overnight. This culture was diluted with 10 volume of Soy medium and shaken further, after 4.5 hours, cultures were divided to 10 ml. portions. Dialyzed antiserum, dialyzed normal serum or saline was added as indicated in the figure and the total volume was made to 12 ml. with saline (0 time). Cultures were shaken further at 30° and formation of amylase was followed. Neutralizing power of antiserum under such condition was tested by mixing the antiserum with Soy culture medium containing the known amount of amylase. 1 ml. of serum neutralized about 25 units of amylase.

DISCUSSION

By the repeated injection of crystalline bacterial amylase into rabbit, the precipitating and neutralizing antibody was easily produced. Maekawa *et al.* reported that the bacterial amylase was ineffective as an antigen, while bacterial amylase oxidized by periodate was effective (*13*). The bacterial amylase preparation employed by them is very similar to that used by us. Their failure of producing antibody is probably due th the small quantity of antigen injected.

The amylase activity was neutralized by the antibody up to about 90 per cent. By the measurement of enzyme activity, the simple and quite accurate determination of the anti-amylase neutralization power of serum could be achieved. This allowed to examine the problems raised from the investigation of amylase formation on one hand, and to study the antigen-antibody reaction quite accurately at relatively low concentration of reagents on the other hand. The latter aspect of the study will be dealt in the subsequent paper (*11*).

As described in the introduction of the present paper, the last stage of amylase formation was proposed to be the conversion of preformed complex precursor to active amylase protein. The possibility that this proposed precursor material might be immunochemically related to amylase protein was examine, but with negative results as described in the present paper. The following possibilities can be considered; (a) the proposed precursor material is not capable of combining with anti-amylase, and (b) the precursor is capable of combining with anti-amylase but exists beeing bound to some structual element of cell in a form not accessible to antibody. The first alternative is more plausible, since the antigenically active point of amylase was shown to be structurally related to the enzymatically active site of amylase (*11*) and the precursor is of course enzymatically inactive. However, the immunochemical approach to the problem of proposed precursor will be continued further.

The use of specific antibody against crystalline amylase allowed the examination of two further problems. First, this bacterium was shown to produce only one and the same enzyme molecule under the various conditions either in the medium or within the cell. This result substantiated the correctness of analysis by the simple activity determination of the mechanism of amylase formation.

The second problem was concerning the effect of antibody on the amylase formation. Various speculations may be conceivable concerning the effects of antibody to the enzyme formation. In the experiment using intact cells, no appreciable effect was recognized on the amylase formation. However, the negative results might be due to the inability of antibody to penetrate through the cell surface and the problem must be reexamined in the sub-cellula system.

SUMMARY

Antiserum produced in rabbits by the injection of crystalline amylase was found to neutralize the enzyme activity. The amount of enzyme

neutralized was proportional to that of antibody added in the region of antigen excess.

Search for the protein, enzymatically inactive but capable of combining with anti-amylase neutralizing antibody, was performed both in cultural media and in cell extract. No positive result was obtained. However, in the course of these experiments, the enzymatic hydrolysate of starch was found to interfere strongly with the amylase-anti-amylase reaction.

Bacillus subtilis strain N was found to produce only one and the same amylase judged from the immunochemical criterion either in the culture media or within the cell.

The effect of antibody on the amylase formation by intact cells was examined. No appreciable effect was observed.

The authors wish to express their gratitude to Prof. S. Akabori for his encouragement and guidance, and to Prof. T. Ogata, in whose laboratory the immunization experiment was performed, far his helpful advice and suggestions, especially on the immunological technique. The authors also wish to thank Mr. K. Kusai for the supply of crystalline amylase preparation.

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AN IMMUNOCHEMICAL STUDY OF MICROBIAL AMYLASE (I)

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The problem raised from our studies on the formation of amylase by *Bacillus subtilis* prompted us to undertake the immunochemical study on the bacterial amylase (*1*). Amylase neutralizing antibody was produced in rabbit serum. Using this antiserum some problems were analysed. During the course of these experiments, it was found that the enzymatic hydrolysate of starch interfered with the neutralization reaction between enzyme and antibody (*1*). Such findings lead to the supposition that the substrate or its products of enzyme and antibody against the enzyme might compete for combination at the same portions of enzyme surface. The interrelationship between the enzyme, its substrate and its antibody is an interesting problem from the view point of both enzymology and immunochemistry. Therefore, the detailed studies were undertaken and the results were presented in this paper.

The advantage to study the antigen-antibody reaction using an enzyme as an antigen was stated by several workers (*2*, *3*). The bacterial amylase is a simple protein, and can be obtained easily in a pure state in large amounts (*4*). The measurement of its activity is simple and quite accurate. Its chemical composition and structure were studied (*5*, *6*). Thus, this enzyme seems to be a suitable material for the study of enzyme immunochemistry. In this paper, some immunochemical properties of bacterial amylase were described.

METHODS

The preparation of antiserum against cystalline amylase of *Bacillus subtilis* N was described in the previous paper (*1*). The amylase activity was usually assayed according to the method of Hagihara (*4*). The ability of samples to interfere with amylase-anti-amylase reaction was measured in a similar way to the serum blocking power as described in the previous paper (*1*), but was expressed as a per cent inhibition of amylase-anti-amylase neutralization reaction according to the following equation:

$$\text{per cent inhibition} = \frac{(\text{N. U. of the serum})\text{-}(\text{N. U. of the serum in the presence of test sample})}{(\text{N. U. of the serum})} \times 100$$

(N. U. = Neutralization unit; see in the previous paper (*1*))

Protein of antigen-antibody precipitate was determined according to the method of Lowry *et al.* using rabbit serum globulin as standard (*7*). Reducing sugar was measured colorimetrically (*8*).

RESULTS

Precipitation Reaction—The neutralization of enzyme activity by antiserum was described in the previous paper. The precipitation reaction was studied quantitatively. The results were presented in Table I (a) and (b). The

Table I (a)

Amount of Precipitate Formed by the Addition of Increasing Amounts of Antibody to a Constant Amount of Amylase

Serum added (*ml.*)	Precipitate ($\mu g.$)	Reagent present in excess	Molecular ratio (Ab/E)*
0	1	E (79 $\mu g.$)	—
0.2	171	E (43 $\mu g.$)	1.56
0.5	585	E (3 $\mu g.$)	1.83
1	878	Ab	2.7
2	878	Ab	2.7
3	942	Ab	3.0

79 $\mu g.$ of amylase dissolved in 0.5 ml. of calcium acetate solution ($M/500$) was mixed with various amounts of antiserum as indicated in the table. Total volume was adjusted to 3.5 ml. with saline. After 4 days at 0°, precipitate was centrifuged, washed once with cold saline and the protein of precipitate was analyzed.

* Following molecular weight were assumed for calculation: γ-globulin (Ab), 165,000; bacterial amylase E, 45,000.

Table I (b)

Amount of Precipitate Formed by the Addition of Increasing Amount of Amylase to a Constant Amount of Antibody

Amylase added	ppt. ($\mu g.$)	Reagent present in excess	Molecular ratio
20	162	Ab	1.9
60	—*	Ab	—
100	672	E (3 $\mu g.$)	1.58
150	672	E (31 $\mu g.$)	1.28
200	654	E (80 $\mu g.$)	1.22
400	523	E (290 $\mu g.$)	1.03
600	423	E (500 $\mu g.$)	0.89

0.5 ml. of antiserum was mixed with 3.0 ml. of calcium acetate solution containing various amounts of amylase as indicated in the Table. The precipitate was analyzed as described in Table I (a).

* Not determined by accident.

molecular ratio of antibody to enzyme at an equivalent point was found to be between 1 and 2. The general behavior was similar to those of other precipitating systems. When antigen was in excess, the amount of precipitate was decreased.

Inhibition of Amylase-Anti-Amylase Neutralization Reaction by Starch Hydrolysate—In the previous paper it was reported that the hydrolytic products of starch with amylase interfered with the amylase-anti-amylase neutralization reaction. It was suggested that the oligosaccharides derived from starch competed with antibody for the combination with enzyme. Further analyses on this point were performed.

First, the effects of varying the concentration of starch hydrolysate and of antiserum on the inhibition of neutralization reaction by starch hydrolysate were examined. Table II summarizes the results obtained. For a given antiserum and enzyme concentration the degree of inhibition of neutralization reaction increased as the concentration of starch hydrolysate increased. On the other hand, for a given concentration of starch hydrolysate and enzyme, the degree of inhibition decreased as the concentration of antiserum increased. Thus, the apparent competitive interaction between starch hydrolysate and antibody in the combining reaction with enzyme was demonstrated.

Table II

Inhibition of Amylase-Anti-Amylase Reaction by Starch Hydrolysate. Effects of Varying the Concentration of Starch Hydrolysate and Antiserum

Exptl. No.	Concentration of starch hydrolysate (%)	Concentration of serum		
		1/10	1/5	1/2
1	10.4	75	33	2
	5.2	74	14	0
2	10.4	91		
	5.2	32		
	1.3	11		

Figures in the table are the per cent inhibition of amylase anti-amylase neutralization reaction by starch hydrolysate. The precedure is described in methods.

Effects of Various Carbohydrates on the Amylase-Anti-Amylase Neutralization Reaction—The hydrolytic products of starch with bacterial α-amylase were shown to inhibit the neutralization reaction. This products are so-called α-dextrins. They contain oligosaccharides with varying chain length including branched-chain oligosaccharides derived from amylopectin and a small amounts of fermentable sugars, glucose and maltose. The question arises which of these various carbohydrates contained in the preparation is the most active inhibitor. Various known carbohydrates were examined on their inhibitory effects on the amylase-anti-amylase neutralization reaction.

Table III summarizes the experimental results of such studies. Inhibition of mono- and di-saccharides were slight. Trisaccharides were more effective in their inhibitory action than disaccharides, and maltotriose, a straight-chain trisaccharides, were more effective than panose, a branched-chain trisaccharide. Dextran, a polysaccharide of mainly α-glucosidic-1,6-linkage, was totally inactive. Thus it was concluded that the inhibitory agents were the poly- (or oligo-) saccharides containing α-glucosidic 1,4-linkages.

TABLE III

Effect of Varius Polysaccharides on the Amylase-Anti-Amylase Reactio ı

Exptl. No.	Polysaccharides	Concentration (%)	Per cent inhibition
1	Maltose	5.2	8
	Glucose	5.2	6
	Lactose	5.2	3
2	Dextran	10.4 5.2	0 0
	Taka amylase-limit dextrin	10.4 5.2	32 26
3	*iso*-Maltose	5.2	4.2
	Maltose	5.2	4.8
	Panose	5.2	7.6
	Maltotriose	5.2	22.1

Effects of Starch and Amylose on the Amylase-Anti-Amylase Neutralization Reaction—In the experiments described in the previous section, the effects of polysaccharides, which gave the positive iodine reaction, could not be examined owing to their intercference with the assay method. Therefore, the amylase reaction was followed by the determination of liberated reducing sugars and the effects of the substrate of enzyme per se on the amylase-anti-amylase neutralization reaction were examined. If the amylase was first brought into contact with antiserum and the substrate starch was added after 30 minutes, the amylase action was greatly inhibited for about 3 hours. On the other hand, if amylase was first brought into contact with the substrate starch and the antiserum was added after 5 minutes, no inhibitory action of antiserum was observed for about 2 hours (Fig. 1). A similar result was obtained, when starch was replaced by amylose, a straight-chain polymer (Fig. 2). Thus, it was conculuded that the substrate of amylase (starch and amylose) can effectively prevent the neutralizing action of antibony. The concentration of substrates used in these experiments was low (1 per cent in final test solution) compared with those of degradation products of starch or known oligosaccharides used in the previous experiments.

Therefore, the inhibitory action of starch or amylose on the neutralization reaction of antibody seems to be much greater than that of its decom-

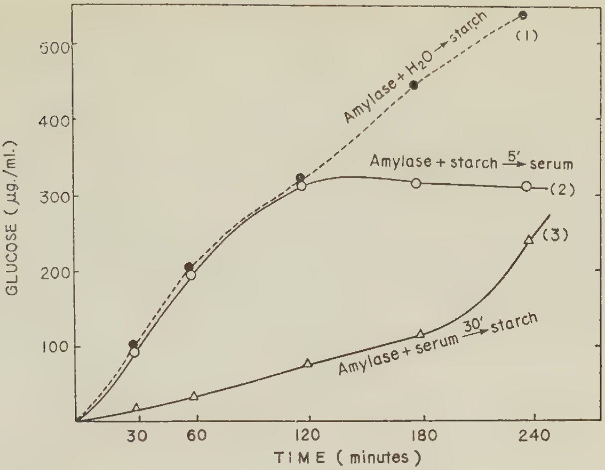

FIG. 1. Effect of adding anti-amylase before and after amylase reaction has started (soluble starch as substrate).

Curve 1: 0.5 ml. of amylase solution (0.2 μg. of amylase) and 0.5 ml. of 0.85 per cent NaCl solution was incubated at 40° After 35 minutes, 1 ml. of 2 per cent starch solution was added (time 0 in the figure).

Curve 2: 0.5 ml. of amylase solution and 1 ml. of 2 per cent strach solution was mixed. After 5 minutes, 0.5 ml. of 50 fold-diluted antiserum was added (time 0 in the figure).

The liberation of reducing groups (expressed as glucose) was followed colorimetrically. The values were corrected for reducing sugars at 0 time and those due to antiserum. Amylase solution was prepared by dissolving crystalline amylase in calcium acetate solution containing 0.02 per cent of sodium azide.

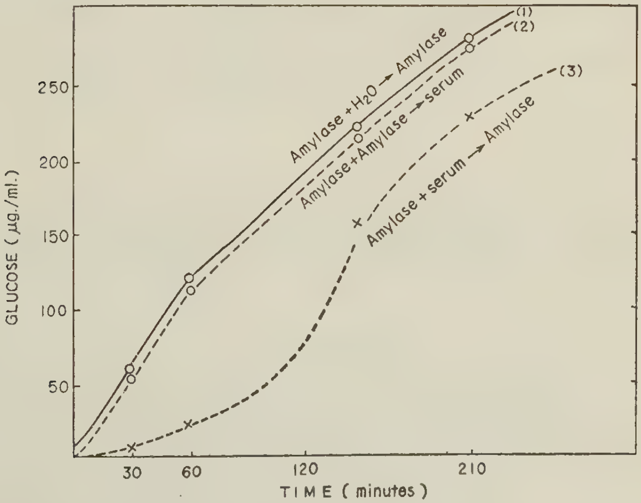

FIG. 2. Effect of adding anti-amylase before and after amylase reaction has started (amylose as substrate).

Experimental conditions were the same as those described in Fig. 1 except that amylose was used instead of soluble starch.

position products. This explains the decline of the curve 2 after 2 hours in Fig. 2. Probably, the substrate starch, which had been effective in preventing the inhibitory action of antiserum, was degraded to small molecular weight oligosaccharides and the inhibitiory action of antiserum became apparent. On the other hand, it can be seen from curve 3 in Fig. 2 or 3 that the amylase once inhibited by antiserum regained its activity after two to three hours of incubation with substrate. This is not due to the inactivation of antiserum, because the degree of the neutralization of enzymatic activity with antiserum does not change during the incubation period of several hours in the absence of substrate (*cf.* Fig. 1 in the previous paper (*1*)). Therefore, the recovery of the amylase activity seems to be due to the displacement of antibody in the antibody-enzyme complex by the substrate. Thus, it was suggested that the substrate and antibody may compete for combination with the same regions of the enzyme molecule.

Inhibition of Amylase-Anti-Amylase Precipitation Reaction by Starch and Starch Hydrolysate—Starch or its hydrolytic products was shown to interfere with the amylase-anti-neutralization reaction. These effects on the precipitation reaction was also examined. Typical data were shown in Table IV. The inhibition of precipitation hydrolytic products were demonstrated.

TABLE IV

Effects of Starch and Its Enzymatic Hydrolysate on the Precipitation Reaction Between Amylase and Anti-Amylase

Polysaccharide added	Time of incubation (*hr.*)	Amount of precipitate (μg. N/*ml.*)	Inhibition due to polysaccharide (%)
—	2	8.4	—
Starch	2	6.7	20.3
—	1	8.5	—
Starch hydrolysate	1	6.4	24.8

0.2 ml. of four-fold diluted serum and 0.4 ml. of 16 per cent of polysaccharide solution was mixed, and 0.4 ml. of amylase solution (containing 10 μg. amylase) was added to those mixture. After the incubation at 40°, the precipitates were collected and their amounts were determined as described in the Table I (a).

DISCUSSION

Both the neutralization reaction and the precipitation reaction between bacterial amylase and its antibody were studied in the present and the previous papers. The method based on the neutrlization reaction is more convenient and economical of both time and material. The problem of interrelationship between enzyme, its substrate (or products) and its antibody was studied in detail based mainly on the neutralieation reaction. It was suggested that the substrate (or products) and antibody might compete for combination with the same protions of enzyme surface. Among many carbohydrates tested, the substance more related to amylose is more effective in preventing the neutralizing action of antiserum. The effectiveness seems to correspond to the affinity to the enzyme as substrate. Thus, the enzymatically active site (as measured by the substrate combining site) was suggested to be the serologically active site. However, the antiserum against bacterial α-amylase cannot inhibit the amylase of fungi and malt (*1*) and no common serologically active structure is demonstrated. The question whether the similar enzymatic activity reflects the similar chemical structure in the portions of enzyme molocule is also unanswered. The solution of the problem on the relationship between chemical structure necessary for the action as an enzyme and that as an antigen must await further studies.

In addition to the neutralization reaction, the precipitation reaction was also prevented by starch or its hydrolytic products. Therefore, the precipitation reaction and the neutralization reaction seem to be the same process of antigen-antibody interaction at least in their initial steps.

An apparent recovery of enzymatic activity of enzyme-antibody complex by substrate (curve 3 in Fig. 2 and 3) presents an interesting problem of reversibility of antigen-antibody union. Though the antigen-antibody union is usually thought not to be freely reversible, the reversibility is dissolved by the excess of antigen. Further studies on the recovery of enzymatic activity by substrate may shed some light on this problem.

Effect of enzyme-antienzyme in reaction is not confined to that presented here. Sometimes, the antiserum precipitates enzyme, but does not inhibit its action (*2*, *9*). In other case, the enzymatic activity is neutralized, but the neutralization seems to be due to a steric hindrance or an " aggregation effect " (*10*). The more specific inhibition of enzymatic activity by antiserum, related to the substrate or coenzyme combining site, is also conceivable as in the case of present study and also in other systems (*11–14*). Unified understanding of such diverse cases is to be developed in the future.

SUMMARY

The precipitation reaction between bacterial amylase and its antibody was studied quantitatively. The molecular ratio of antibody to enzyme in the precipitate was calculated.

The enzyme-anti-enzyme neutralization reaction was found to be inhibited by substrate and antiserum on this phenomenon suggested an apparent competitive relationship between starch hydrolysate and antibody.

Various known carbohydrates were examined on their inhibitory effects on the amylase anti-amylase neutralization reaction. Inhibitory effect was correlated with the structure of straight chain poly-(or oligo) saccharides containing α-glucosidic-1,4-linkage.

Inhibitory effect of starch and amylose on the amylase-anti-amylase neutralization reaction was studied kinetically. An apparent competitive

relationship between substrate and antibody was also observed.

In addition to neutralization reaction, the precipitation reaction between enzyme and its antibody was also found to be inhibited by its substrate (or products).

It was suggested that the substrate (or its products) and antibody might compete for combination at the same portion of the enzyme surface.

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METABOLISM OF D-GLUCOSAMINE

III. ENZYMIC DEGRADATION OF D-GLUCOSAMINIC ACID

Recently, the author reported the formation of glucosaminic acid (GAA) by *Pseudomonas fluorescens* and its extract from D-glucosamine (*1*). It may perhaps be the first case that GAA has appeared in the metabolic patterns. Imaizumi (*2*) reported the degradation by mammals and bacteria of GAA, but its mode of degradation is still obscure.

The author isolated a species of soil bacterium which utilized GAA as a sole source of carbon and nitrogen. This bacterium consumed about 5 atoms of oxygen per molecule of GAA, but no oxygen when 2-ketogluconic acid was used as substrate. The degradation of GAA may be a non-adaptive one (Fig. 1).

By disintegrating cells with silica sand and centrifuging the cell debris (20,000 r.p.m.), a clear supernatant was obtained. This crude enzyme solution did not show any oxygen consumption for GAA, but degraded it forming ammonia and ketonic acid. Addition of ATP showed almost no effect for the formation of ammonia and total ketonic acid, but greatly accelerated the pyruvic acid formation (Table I).

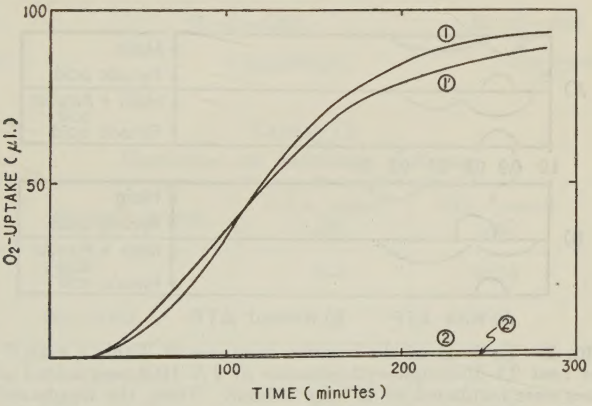

FIG. 1. Cells were harvested and washed once after the cultivation of two days on the same media as the preceding series of this article, but in case of ①' and ②' the media contained additional GAA (0.2 per cent).

Complete system: 2.4 ml. of cell suspension, 1.0 ml. of $M/15$ phosphate buffer (pH 7.0), 0.4 ml. (1.6 μM) of substrate (①, ①'; GAA, ②, ②'; 2-ketogluconic acid) and 0.2 ml. of 10 per cent KOH (center well). Controls lacked substrate. Experiments were carried at 30°.

TABLE I

Degradation of GAA by Crude Extracts

ATP	GAA added (μM)	NH_3 formed (μM)	Total ketonic acid formed (μM)	Pyruvic acid formed (μM)
+	3.8	4.6	3.1	0.80
−	3.8	4.5	2.6	0.026

Complete system: 3.6 ml. of crude extract, 1.2 ml. of NaF (final concentration, *ca.* 3×10^{-3} *M*), 1.2 ml. of $MgSO_4$ (60 μM), 1.2 ml. of ATP (60 μM), 4.2 ml. of *M*/15 phosphate buffer (pH, 7.0) and 1.2 ml. of GAA (50 μM) in the same phosphate buffer. After the incubation of the above mixtures (controls lacked substrate, GAA) for 5 hours at 30°, each 0.6 ml. of 40 per cent metaphosphoric acid solution was added to the reaction mixtures, and after centrifugation the supernatants obtained were analysed.

Methods of analysis: NH_3; Conway-Nessler method (*5*). Total ketonic acids (calculated as pyruvic acid); Semicarbazide method (*4*). Pyruvic acid; Friedemann-Haugen method (*6*).

Chromatograms of the reaction products with 2,4-dinitrophenylhydrazine showed the formation of pyruvic acid and another ketonic acid (Fig. 2).

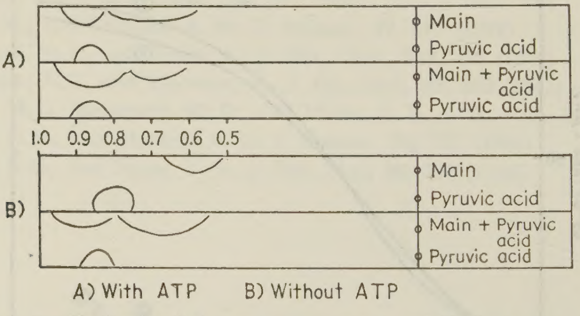

FIG. 2. To 7 ml. of the reaction mixtures of Table I, each 7 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 *N* HCl was added and the mixtures were incubated at 40° for 1 hour. Then, the incubated solutions were extracted with ethylacetate and the hydrazones of ketonic acids in the ester layers were extracted with 10 per cent Na_2CO_3 solution. The Na_2CO_3 solutions were acidified by adding HCl, the acidified solutions were re-extracted with ethylacetate, the ester layers were concentrated and chromatographed.

Controls contained no detectable hydrazone. Moving phase system: *n*-Butanol-acetic acid- water (4 : 1 : 1).

It has been reported that β-hydroxy-α-amino acids (serine and threonine) are deaminated by their dehydrases to form β-deoxy-α-ketonic acids (pyruvic acid and α-keto-butyric acid, respectively) (*3*). On the other hand, 6-phospho-gluconic acid is dehydrated to 2-keto-3-deoxy-6-phospho-gluconic acid and the latter is further degraded to pyruvic acid and 3-phospho-glyceraldehyde by the splitting enzyme (*4*). In the detection of ketonic acid, 2-keto-3-deoxy-

6-phospho-gluconic acid is positive in total ketonic acid determination, but is nagative in Friedemann-Haugen's pyruvic acid determination (*4*).

These reports and the results mentioned above suggest the Scheme 1 for the possible degradation pathway of GAA.

SCHEME 1

A Supposed Metabolic Pathway of GAA

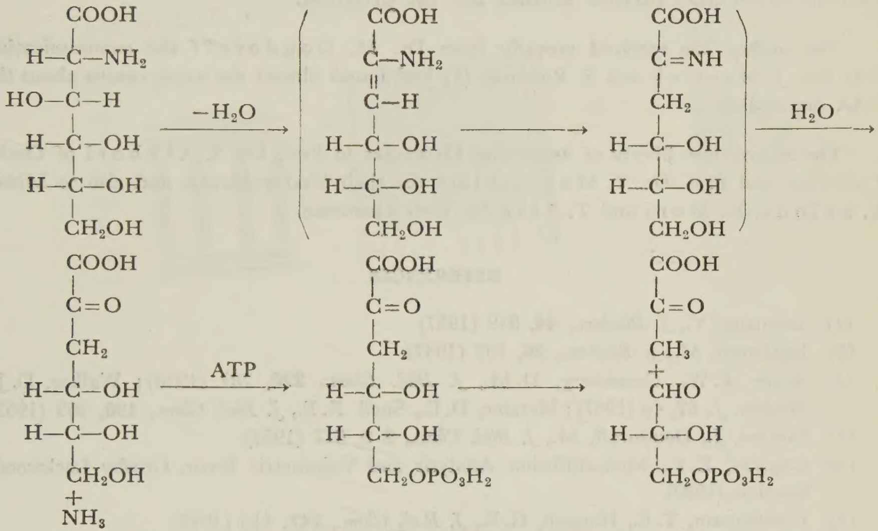

TABLE II

Deactivation and Reactivation of Enzyme

		Enzyme system	GAA added (μM)	NH_3 formed (μM)	Total ketonic acid formed (μM)
(A)	(1)	DE	3.0	0.53	0.39
	(2)	DE+SHE	,,	1.8	1.1
	(3)	DE+SHE (Norit)	,,	0.67	0.45
(B)	(1)	DE	3.0	0.97	0.58
	(2)	DE+ATP*	,,	1.0	0.55
	(3)	DE+ATP+PAL	,,	2.1	1.2

DE; Dialysed enzyme. SHE; Supernatant of heated crude enzyme (100°, 8 minutes). PAL; Pyridoxal.

(A) 0.6 ml. of DE, 0.5 ml. of $M/15$ phosphate buffer (pH 7.0), 0.2 ml. of GAA (6 μM), and 0.6 ml. of water. In case of (2) or (3), water was replaced by 0.6 ml. of SHE or SHE (Norit) respectively.

(B) 0.6 ml. of DE, 0.6 ml. of $M/15$ phosphate buffer (pH 7.0), 0.2 ml. of GAA (6 μM), 0.1 ml. of ATP (5 μM), 0.1 ml. of $MgSO_4$ (5 μM), 0.2 ml. of NaF (final conc., 3×10^{-2} M) and 0.1 ml. of PAL (2 μM). In case of (1) or (2), (PAL+$MgSO_4$+NaF+ATP) or PAL was replaced by water respectively.

30°, 3 hours. Deproteinization with 0.1 ml. of 40 per cent metaphosphoric acid.

* Kindly supplied by Dr. Suda, Osaka University.

The activity of the crude extract was greatly decreased by dialysis at 0°, but it was partially restored by addition of the heated extract or pyridoxal and ATP. The co-factor in the heated extract could be adsorbed to Norit (Table 2). Thus, the first step reaction may be catalysed by a pyridoxal phosphate-depending enzyme.

Further, the ketonic compound was isolated as a potassium salt. Its identification and further studies are on progress.

The author has received recently from Dr. M. Doudoroff the communication that Drs. J. Merrick and S. Roseman (*7*) had found almost the same results about the GAA metabolism.

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